Chemotherapy and Drug Targeting in the Treatment of Leishmaniasis

Final Report

Linda L. Nolan

January 31, 1993

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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Leishmaniasis, a disease caused by protozoan parasites of the Leishmania spp., is one of the major public health problems currently affecting humanity. Therapeutic agents for this disease is either ineffective or toxic. The purpose of this work is to aid in the development of an effective, non-toxic treatment for leishmaniasis.

The objective of this research was to isolate and characterize unique leishmanial enzymes (DNA polymerase) and to test promising antileishmanial compounds for toxicity against human CEM T_4 cells.

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TITLE PAGE

Title of Study (120 characters) Chemotherapy and Drug Targeting in the Treatment of Leishmaniasis.

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Abstract

(type within outline; approximately 200 words)

Leishmaniasis is a disease caused by protozoan parasites, which are major public health problems currently affecting humanity. Therapeutic agents for these diseases are either ineffective, toxic or if effective, parasite resistance to them is developing.

The purpose of this work is to aid in the development of an effective, non-toxic treatment of leishmaniases.

The objectives of this research were the following:

1. To isolate and characterize unique enzymes or requirements of DNA synthesis of these protozoan parasites.

2. To target this critical enzyme system. To identify an inhibitor which is non-toxic to host cells which will target the unique enzyme system of the parasite.

3. To test promising compounds (sent by the Walter Reed Institute of Research) against *Leishmania sp.* to determine their anti-leishmanial effect.

4. to test promising anti-leishmanial compounds (sent by WRAIR) in an *in vitro* CEM T_4 cell system to determine possible host toxicity and the potential of the compounds to compromise the immune response.

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MILITARY SIGNIFICANCE

The need for leishmanicides cannot be overemphasized. At present chemotherapy is dependent on a relatively small number of synthetic drugs. Resistance has been reported to occur against all these drugs and development of resistance to one compound is often accompanied by cross-resistance to others. In the chemotherapy of visceral and cutaneous leishmaniasis, the choice of drugs is very limited and success of a particular drug appears to vary from locality to locality presumably due to strain differences in Leishmania.

To date the logical design of antiparasitic drugs has proved largely unsuccessful with the exception of purine metabolism in protozoa. While mammalian cells are capable of de novo synthesis of purines, many parasites do not synthesize purines but use salvage pathways. Analogues inhibiting key enzymes in purine pathway should, therefore, provide novel therapeutic agents. Purines and pyrimidines serve not only as precursors of RNA and DNA, but also as stores of high energy phosphate, constituents of certain coenzymes, and modulators of various enzymatic reactions. In view of this vital role, intervention of their metabolism will have profound effects on the organism.

To date there is no safe, effective, and quality-controlled antiparasitic vaccines. Membrane antigens differ from one species to another and during the course of infection, making the production of a useful vaccine very difficult.

The elucidation of the biochemical mode of action of promising compounds and the identification of unique enzyme systems will permit the logical design of more effective derivatives and also will provide insight on the mechanism of drug resistance. This information may allow a therapy program to be developed which would decrease or eliminate the problem of drug resistance.

Targeting of already promising compounds may increase the efficacy of these compounds for the various disease states of leishmaniasis and be more cost effective than the development of more than one drug.

Targeting will also allow the reduction in toxicity of certain compounds, and also be more cost effective since less drug should be required.

POTENTIAL RELEVANCE OF PROPOSED RESEARCH TO WRAIR MISSION NEEDS

RESEARCH ON LEISHMANIASIS:

1. Leishmaniasis is endemic to 80 countries, with 350 million people at risk, 12 million infected, and an annual incidence of 3-4 million.

2. Drug resistance, manifested by treatment failure, is reported in virtually all endemic areas.

3. Epidemics including several hundred thousand patients with tens of thousands of deaths were reported in 1990 from the Sudan and India.

MILITARY IMPACT/RELEVANCE:

1. Leishmaniasis are endemic to most areas of strategic concern (e.g. the Middle East, Asia, southern Europe, Africa, Central- and South America).

2. Certain leishmanial diseases are fatal or lead to severe disfigurement if untreated.

3. Prevention/treatment of leishmaniasis in deployed and returning soldiers is a public health concern because the sandfly vector(s) inhabit the southern half of the United States.

INTRODUCTION

Leishmaniasis is caused by protozoan parasites of the Order Kinetoplastida: Family-Trypanosomatidae. The disease is estimated to affect 12 million people in Third World countries. Leishmania extracellular forms (promastigotes) are injected into human skin during bites by the sandfly vector. Promastigotes are phagocytized by reticuloendothelial cells, within which the parasites transform into intracellular amastigotes. Human disease results from multiplication of amastigotes within macrophages. Present therapy with pentavalent antimony is potentially toxic, and often ineffective. One rationale for searching for alternative treatment is to identify a unique enzyme system and to target this system for chemotherapeutic exploitation.

Many of the enzymes involved in the synthesis of nucleic acids of the parasitic protozoans have been found to be unique, and for this reason we have begun studies to compare the DNA synthetic enzymes of parasitic protozoa to the mammalian polymerases. During this research year we rested a number of compounds for Walter Reed Institute of Research (WRAIR) for antileishmanial activity .

We have developed a human CEM T_4 in vitro assay to determine the toxicity of promising antileishmanial compounds for host cells. Because T_4 cells are extremely important in eliciting the immune response, it is of profound importance that these cells are not compromised during chemotherapy of a parasitic disease Use of our assay system can save WRAIR expensive and time-consuming *in vivo* animal testing and provide critical data on promising compounds.

We continued our investigations of the DNA polymerases of the leishmanial parasite because (1) these enzymes are unique from host enzymes and (2) they are extremely important in parasite survival. Compounds which are shown to be inhibitory to leishmanial DNA polymerases and exhibit low toxicity in the CEM T_4 system are potential candidates as therapeutic agents.

We investigated the properties of the S-adenosylmethionine synthetase of L. mexicana because this enzyme is responsible for the methylation of a variety of important biological molecules and methylation of DNA which has been implicated in gene expression. If biochemical differences can be found between parasitic and host enzymes this crucial enzyme could be exploited for chemotherapeutic purposes.

METHODS

Cultures of parasitic protozoa. Promastigotes of *Leishmania mexicana* Walter Reed strain 227, were maintained in this laboratory in tissue culture flasks containing the defined medium of Steiger and Black (1) supplemented with 5% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, New York) and 50 mg/L gentamycin. The cells were grown at 26°C and subcultured weekly.

Cultures of T_4 cells. Human lymphocyte CEM T_4 cells were obtained from the Department of Pharmacology at the University of Massachusetts Medical Center. They were cultured in tissue flasks containing RPMI 1640 medium (Sigma Chemical Co., St. Louis, Missouri) supplemented with 0.1% sodium Bicarbonate, 5% heat-inactivated fetal calf serum, and 50 mg/L gentamycin. Cultures were incubated in a 5% CO_2 chamber at 36°C subcultured semi-weekly.

Assay inoculum. *Leishmania sp.* and T_4 cells were diluted with fresh medium 24 h prior to use to ensure a log phase culture. The inoculum was standardized at the start of the assay with a spectrophotometer (Spectronic 21, Bausch & Lomb, Rochester, New York) in order to eliminate variations caused by different concentrations of cells growing at varying rates, and thus being inhibited differentially owing to cell concentration. Cell stock was centrifuged in a microcentrifuge at 200 - 400 g for 3 minutes and resuspended in fresh medium to give an initial cell concentration of approximately 5 X 105/ml in the test wells.

Model for microwell plate assay procedure. Assays were performed in Corning sterile covered polystyrene 96-well round bottom tissue culture plates that were not tissue culture treated. This is very important because cells will adhere to the surfaces of treated wells, and the absorbance readings will be inaccurate. All wells contained a uniform total volume for the assay.

Blank wells contained equal volumes of medium and sterile deionized double-distilled water. Control wells received medium, water, and inoculum, while test wells received the increasing amounts of test compound replacing water. Six replicates of each level of test compound were made. The standardized inoculum was stirred gently, under aseptic conditions, in a deep Petri dish, and suitable aliquots were pipetted into all but the blank plate wells. Absorbance readings were taken on a microplate

spectrophotometer (Microplate Reader, Model MR 600, Dynatech Laboratories, Inc., Alexandria, Virginia) set in single wavelength mode with a suitable filter (490, 660 nm). The plate was shaken on a Vortex Genie-2 fitted with a 6-inch (15-cm) platform head containing a 96-well plate insert in order to ensure suspension of the cells just prior to reading the absorbance. Microwell plates containing leishmania were incubated in a 26°C incubator and read at 0, 24, 48, and 72 h. Microwell plates containing T_4 cells were incubated at 37°C with 5% CO_2 for the desired time. Toxicity studies were usually monitored at O and 72 h. Cellular toxicity was measured by determining the IC_{50} (that concentration of an agent causing 50% inhibition compared with controls.)

To determine whether trubidity, observed photometrically in the microwells, would have a direct relationship to the cell concentration, we added several dilutions of cultures of Leishmania cells and human CEM T_4 cells to microwells. Absorbance was measured with a microplate reader, and the well contents were counted on the Coulter counter, which had been calibrated against a hemocytometer for each cell line. As a further check of the accuracy of this rapid method, we compared the IC_{50} of pentamidine, a known antileishmanial agent [2], by the microwell methods and the test tube method.

Test tube assay procedure. The assay procedure, a modification of the method of Kidder and Dewey [3], has been used for drug screening regularly in this laboratory. Scratch-free pyrex screw cap tubes (16 X 150 mm) were selected to match as closely as possible for use in the assay. The medium of Steiger and Black, supplemented with test compounds or water in a total volume of 5 ml, was used. The tubes (in triplicate) were incubated with loose caps in a slanted position (5°) in an incubator at 26°C for 72 h. The tubes were vortexed before reading the absorbance at 660 nm, by use of a spectrophotometer equipped with a test tube chamber.

Cell counts using a Coulter counter. Aliquots from wells were counted at time 0 and 72 h with Model ZF Coulter counter (Coulter Electronics, Hialeah, Florida) with settings of 1 /amp = 0.707 and 1/aperture current = 2 for the protozoan assays and 0.707, 16, respectively, for the T_4 lymphocyte assays.

Cell culture conditions for enzyme isolations. Promastigotes of Walter Reed strain 227 were used in these experiments. This strain has been previously identified as *Leishmania mexicana amazonensis* (J. Decker-Jackson and P. Jackson, personal

communication) and was obtained from the Leishmania Section of the Walter Reed Army institute of Research. Promastigotes, were grown in brain heart infusion medium containing 37 g of brain heart infusion medium (Difco Laboratories, Detroit, Mich.) liter of water^{-1}, 10% heat inactivated serum, and 26 mg of hemin ml^{-1}. Cells were grown at 26°C in 2,000-ml wide Fernbach flasks containing 250 ml of brain heart infusion medium. Cells were harvested after 4 days during the exponential growth phase. The cell density was 4×10^7 to 6×10^7 cells ml^{-1}.

Protein assays. Protein concentrations were determined by either the dye-binding method (Bio-Rad laboratories, Richmond, Calif.) or a modified method. The modified method was performed in 96-well microplates by adding 80 ml of Bio-Rad dye and 20 µl of a column fraction. The plate was them read in a Dynatech 600 miroplate reader at 575 nm.

Isolation and assay of S-Adenosylmethionine Synthetase. Using the method of Hoffman and Kunz (4), we optimized our enzyme assay for L. *mexicana* 227 promastigotes. Methionine adenosyltransferase activity was measured at 35°C in 100 ml of a standard assay mixture containing 150 mM KCl, 20 mM $MgSO_4$, 5 mM, l dithiothreitol, 50 mM Tris (pH 7.5), 5 mM ATP, and lO µM L- [^{14}C]methionine. The cationic [^{14}C]adenosylmethionine formed was isolated by spotting 80-µl portions of reaction mixtures on 2.3-cm- diameter disks of Whatman P81 cellulose phosphate cation-exchange paper, removing unreacted methionine by washing in a beaker of cold 0.1 M ammonium formate (pH 3.0), once with 95% ethanol, and once with ether. [^{14}C]-adenosylmethionine was quantified by liquid scintillation counting of dried disks under 5 ml of Fisher Scint Verse II.

SAM synthetase was isolated by suspending 8 g of pelleted L. *mexicana* 227 cells in buffer containing 50 mM Tris (pH 7.5), 10 mM MSG_4, 1 mM EDTA, and 1 mM dithiothreitol. The cells were sonicated three times for 15 s each time, and the cell suspension was centrifuged at 4°C for 90 minutes at 40,000 X g in an SW 55 Ti rotor. The cell extract (5.3 ml) was applied to a DEAE-cellulose column, and the above buffer was passed through the column until the A_{280} was less than 0.1. the enzyme was then eluted with a linear gradient of KCl (0 to 0.3M) in a volume of 80 ml.

COMPOUND TESTED AGAINST <u>LEISHMANIA MEXICANA</u> 227 (IC_{50} uM)

	WR_NUM	Name	Date	Mode of Action	Approved for Human Use	IC50 (uM)	IC50 (ug/ml)	Max. % Inhib.	Highest Conc. Tested(uM)	Comments
65105	WR 240611 AA					164.00				
65141	WR 263627 AA					165.00				
64911	WR 183751 AA							26.6	570	
64920	WR 184362 AA							13.7	500	
65089	WR 221656 AA							34.3	1000	
65150	WR 249868 AA							27.1	670	
65169	WR 249909 AA							26.5	670	
65178	WR 249941 AA							35.8	1000	
64831	WR 153335 AA							0	400	
64840	WR 171304 AA							0	400	
64859	WR 171333 AA							0	400	
64877	WR 182971 AA							0	400	
64886	WR 182968 AA							0	400	
64902	WR 183750 AA							0	1000	
64957	WR 217246 AA							0	500	
64975	WR 218555 AA							0	375	
64984	WR 218421 AA							0	375	
64993	WR 218418 AA							0	500	
65007	WR 218413 AA							0	500	
65025	WR 220048 AA							0	500	
65034	WR 220033 AA							0	500	
65114	WR 040320 AB							0	1000	
65123	WR 244633 AB							0	250	
65187	WR 249940 AA							0	1000	
64895	WR 183119 AA							0	1000	stimulation
64939	WR 184358 AA							0	800	stimulation
64948	WR 185204 AA							0	1000	stimulation
64966	WR 218368 AA							0	1000	stimulation
65016	WR 219984 AA							0	500	stimulation, precipitate noted in assay tubes
65043	WR 220001 AA							0	1000	stimulation
65052	WR 221235 AA							0	1000	stimulation
65070	WR 222064 AA							0	1000	stimulation
65098	WR 230839 AA							0	1000	stimulation
65132	WR 249721 AA							0	500	stimulation, precipitate noted in assay tubes
	WR 2446							0	120	stimulation
		Azithromycin						25	1000	
		Azidothymidine						0	900	stimulation
		Dideoxycytidine						0	1000	

	WR_NUM	Name	Date	Mode of Action	Approved for Human Use	IC50 (uM)	IC50 (ug/ml)	Max. % Inhib.	Highest Conc. Tested(uM)	Comments
		Dideoxyinosine						0	1000	
		Pentamidine isethionate				42.00				
		Sulfamethoxazole						0	500	
		Sinefungin, Sigma 1				0.03				Sigma 1 - frozen sample, thawed
		Sinefungin, Sigma 2				0.24				Sigma 2 - Freshly prepared
58706	WR 254847 AB	Sinefungin, Walter Reed				0.21				
		Sinefungin, Calbiochem				0.24				
		Elephant garlic (protein extract)					12.5			
		Formycin B		RNA, protein synthesis	No			0	0.04	stimulation
		Cyclic Formycin A monophosphate		Unknown, Growth Inhibitor	No			0	0.06	stimulation
		Allopurinolriboside		RNA, protein synthesis	Now in trial			0	190	stimulation
		4-Aminopyrazolo-pyrimidine		RNA, protein synthesis	No			0	76	stimulation
		N^6-Methylaminopurine-9-ribofuranoside		Guanase, Adenine deaminase Nucleoside hydrolase Transport purines	No					6.5uM Ki for Guanase
		6-Methylaminopurine-9-ribofuranoside		Transport of Nucleosides	No	250.00				
		Hypoxanthine-9-beta-D-arabinofuranoside		Transport of Inosine	No	110.00				
		6-Methlypurine		Transport of Nucleosides	No	75.00				
		6-Methlyoxypurine-riboside		Transport of Adenosine	No	75.00				
		6-Mercaptopurine-riboside		Transport of Nucleosides	No	75.00				
		Adenosine, N^6-cyclohexyl		Transport of Adenosine	No	75.00				
		8-Phenyltheophylline		Transport of Nucleosides		75.00				
		2-Mercaptopyrimidine		Growth Inhibition	No	250.00				
		5-Flouorouracil		Growth Inhibition	Yes	300.00				
		4-Mercapto-2-pyrazolo [3,4-d] pyrimidine		Growth Inhibition	No	750.00				
		Arachidonic Acid				350.00				
		Eicosapentaenoic Acid				660.00				
		Linoleic Acid				600.00				
03027	WR 268613 AA	no name								insoluble, not tested
03009	WR 268612 AA	no name								insoluble, not tested
02904	WR 268603 AA	no name								insoluble, not tested
02931	WR 268605 AA	no name								insoluble, not tested
02959	WR 268607 AA	no name								insoluble, not tested
02986	WR 268610 AA	no name								insoluble, not tested
03045	WR 268614 AA	no name								insoluble, not tested
03054	WR 268615 AA	no name								insoluble, not tested
59588	WR 268317 AA	1,2-Dimethyl-3-hydroxypyrid-4-one				70.00				
21100	WR 243251 AC	7-Chloro-3-(2',4'-dichlorophenyl)-1-[[33'-(dimethlamino)propyl]imino]-1,2,3,4-tetrahydro-9(10H)acridinone				178 (mM)				
29759	WR 250548 AB	(S)-7-Chloro-3-(2,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-[[3-(dimethylamino)propyl]imino]-9-acridinol								insoluble, not tested
34170	WR 250547 AB	(R)-7-Chloro-3-(2,4-dichlorophenyl)-1,2,3,4-tetrahydro-1-[[3-(dimethylamino)propyl]imino]-9-acridinol						16	150	

N	WR_NUM	Name	Date	Mode of Action	Approved for Human Use		IC50 (uM)		IC50 (ug/ml)	Max. % Inhib.	Highest Conc. Tested(uM)		Comments
L 56390	WR 182393 AB	1-(3,4-Dichlorophenyl)-3-(1-isopropyl-4,5-dioxo-2-imidazolidinylidine)-guanidine								8	292		
M 02995	WR 268611 AA	no name											insoluble, not tested
M 02997	WR 268602 AA	no name											insoluble, not tested
M 02959	WR 268607 AA	no name											insoluble, not tested
M 03063	WR 268616 AA	no name											insoluble, not tested
M 03016	WR 257601 AB	no name											insoluble, not tested
M 02986	WR 268601 AA	no name											to be tested
M 02913	WR 268604 AA	no name											insoluble, not tested
M 02940	WR 268606 AA	no name											insoluble, not tested
M 02977	WR 268609 AA	no name											insoluble, not tested
M 08608										58.2	1000		test against higher concentration
K 73252	WR 238605 AC	8-[(4-Amino-1-methylbutyl)amino]-2,6-dimethoxy-4-methyl-5-(3-trifluoromethylphenoxy)quinoline succinate											compound binds and PPT media components
K 01845	WR 006026 AF	6-Methoxy-8-(6-diethylaminohexylamino)lepidine, dihydrochloride					1 (mM)						
M 04186	WR 242511 AD	8-[(4-Amino-1-methylbutyl)amino]-5-(1-hexyloxy)-6-methoxy-4-methylquinoline DL-Tartarate					1.25 (mM)						
M 08175	WR 269097 AA	3-Deazaaristeromycin								22.3	1000		test against higher concentration
M 07918	WR 268970 AA	3-Deazaneplanocin,hydrochloride,hemihydrate								28.6	1000		test against higher concentration
3-1									25				tested against L. max. 222 resulting in an IC50 of 25 ug/ml
3-3								<<	10				tested against L. max. 222 result-ing in an IC50 of <<10
-6a										32	50 ug/ml		
-55										0	50 ug/ml		

COMPOUNDS TESTED AGAINST HUMAN CEM T_4 CELLS (IC_{50} uM)

BN	WR_NUM	Name		IC50 (uM)	IC50 (ug/ml)		IC25 (uM)	Comments
		Sinefungin		11000	28.8			
		DFMO		10400	57.1			Eflornithine (DL-alpha-difluoromethylornithine)
		Sulfamethoxazole		3900	16.7			
	WR 2446			2270	5.8			
		Naltrexone		1656	4.4			An Immune Modulator that stimulates CD4+ cells in vivo.
		Flucytosine	>	1500	11.6			
	WR 183750			1020	2.2			
		TMP-SMX		52.6	0.18			*Trimethoprim* - Sulfamethoxale (Proloprim or Bactrim) in 1:19 ratio.
		Acyclovir		540	2.4			Zovirax
		Dapsone	>	500	2			
		Trimethoprim		420	1.4			
		AZT		220	0.8			Zidovudine
		Pentamidine		7	0.012			
		2'3'-Dideoxycytidine		6	0.028			
		Ketoconazole		2.3	0.004			
BL 59588				460				
BL 21100				92				
BL 34170				26				
BL 56390				96				
		Allopurinol Riboside	>	12300				
		Meglumine antimoniate (Glucantime)	>	12000				Meglumine antimoniate
		9-deazainosine		4000				
		Cyclic sinefungin	>	3000				
		Cordycepin		3000				
		SIBA		250				5-deoxy-5(isobutylthio)-3-adenosine

BN	WR_NUM	Name		IC50 (uM)	IC50 (ug/ml)		IC25 (uM)	Comments
		Formycin B		13				
		SRTC		13				5'-o-sulfamoyl-1-B-D-ribofuranosyl triazole-3-carboxamide
		7-deazainosine		12				
		Formycin A	<	8				
		Allium sativus (Garlic)			11			11 ug protein/ml (0.001 of a clove/ml) This is made from a crude extract of raw garlic which is diluted in double-distilled water and sterile-filtered.
ZP 64831	WR 153335 AA		>	1000			390	
ZP 64840	WR 171304 AA			430			253	
ZP 64859	WR 171333 AA		>	1000		>	1000	
ZP 64877	WR 182971 AA			1004			325	
ZP 64886	WR 182968 AA			379			240	
ZP 64895	WR 183119 AA		>	1000		>	1000	
ZP 64902	WR 183750 AA		>	1000			317	
ZP 64911	WR 183751 AA			26			8	
ZP 64920	WR 184362 AA			927			259	
ZP 64939	WR 184358 AA		<	10		<	10	
ZP 64948	WR 185204 AA		>	1000		>	1000	
ZP 64957	WR 217246 AA			354			116	
ZP 64966	WR 218368 AA			218			81	
ZP 64975	WR 218555 AA			300			121	
ZP 64984	WR 218421 AA			927			510	
ZP 64993	WR 218418 AA		>	1000		>	1000	
ZP 65007	WR 218413 AA		>	1000			605	
ZP 65016	WR 219984 AA			422			10	
ZP 65025	WR 220048 AA			1000			44	
ZP 65034	WR 220033 AA			68			16	
ZP 65043	WR 220001 AA			388			10	
ZP 65052	WR 221235 AA			587			58	
ZP 65070	WR 222056 AA		>	1000		>	1000	
ZP 65089	WR 221656 AA			100			10	
ZP 65098	WR 230639 AA		>	500			50	
ZP 65105	WR 240811 AA		<	10		<	10	
ZP 65114	WR 040320 AB		>	1000		>	1000	
ZP 65123	WR 244633 AB		>	1000			410	

BN	WR_NUM	Name		IC50 (uM)	IC50 (ug/ml)		IC25 (uM)	Comments
ZP 65132	WR 249721 AA			95			28	
ZP 65141	WR 263527 AA		>	1000		>	1000	
ZP 65150	WR 249868 AA			851			373	
ZP 65169	WR 249909 AA			309			133	
ZP 65178	WR 249941 AA			546			218	
ZP 65187	WR 249940 AA		>	1000			529	
		7-Deazaaristeomycin		19.6				
		Cyclic Formycin A		250				
		Oxyformycin B		2000				
		Sangivamycin		6.4				
		Spermidine	<	100				
HB-1								25% inhib. at 5 ug/ml
HB-3								21% inhib. at 5 ug/ml
PN-6a								22% inhib. at 5 ug/ml
DL-55								74% inhib. at 5 ug/ml

The Toxicity of 34 Adenosine Analogs to Human CD4 T-Lymphocytes as measured in ug/ml.

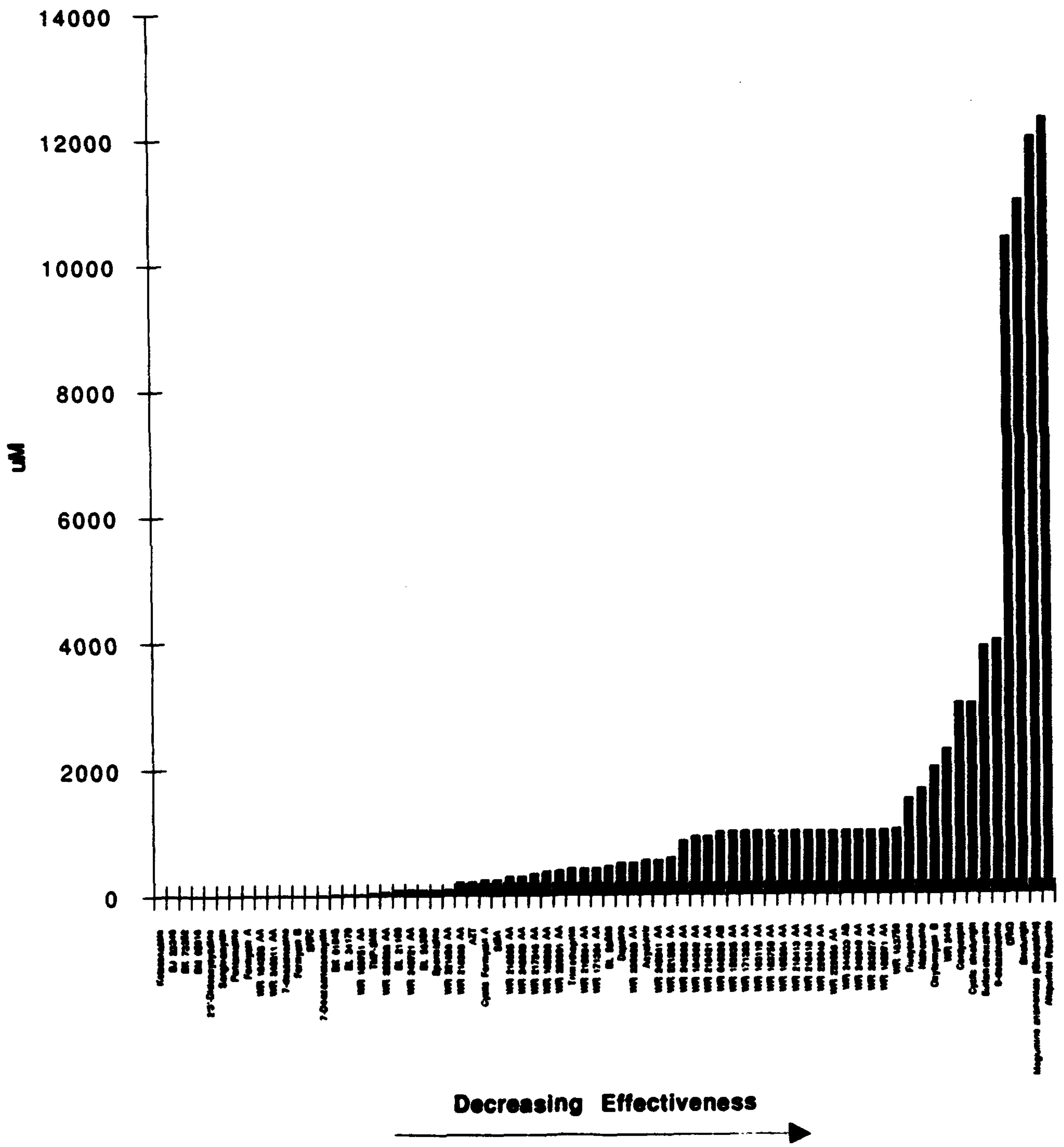
Compound: ZP Number	WR Number	ID_{50} (ug/ml)	ID_{25} (ug/ml)
ZP 64939	WR 184358 AA	<4	<4
ZP 65105	WR 240811 AA	<4	<4
ZP 64911	WR 183751 AA	10	3
ZP 65034	WR 220033 AA	25	6
ZP 65089	WR 221656 AA	34	3
ZP 65132	WR 249721 AA	43	13
ZP 64966	WR 218368 AA	77	28
ZP 64975	WR 218555 AA	121	44
ZP 64886	WR 182968 AA	122	77
ZP 65043	WR 220001 AA	131	3
ZP 65169	WR 249909 AA	131	56
ZP 64840	WR 171304 AA	145	85
ZP 64957	WR 217246 AA	163	53
ZP 65016	WR 219984 AA	181	4
ZP 65052	WR 221235 AA	222	22
ZP 65178	WR 249941 AA	227	91
ZP 64831	WR 153335 AA	>214	83
ZP 65098	WR 230639 AA	336	17
ZP 64877	WR 182971 AA	347	112
ZP 64920	WR 184362 AA	352	98
ZP 65150	WR 249868 AA	353	155
ZP 65187	WR 249940 AA	358	189
ZP 65025	WR 220048 AA	371	16
ZP 65123	WR 244633 AB	388	159
ZP 65007	WR 218413 AA	>326	197
ZP 64902	WR 183750 AA	>470	149
ZP 64984	WR 218421 AA	510	143
ZP 64859	WR 171333 AA	>295	>295
ZP 64993	WR 218418 AA	>303	>303
ZP 64895	WR 183119 AA	>320	>320
ZP 64948	WR 185204 AA	>341	>341
ZP 65070	WR 222056 AA	>343	>343
ZP 65114	WR 040320 AB	>354	>354
ZP 65141	WR 263527 AA	>374	>374

Enzyme Studies on DNA Polymerases from *L. mexicana* 227

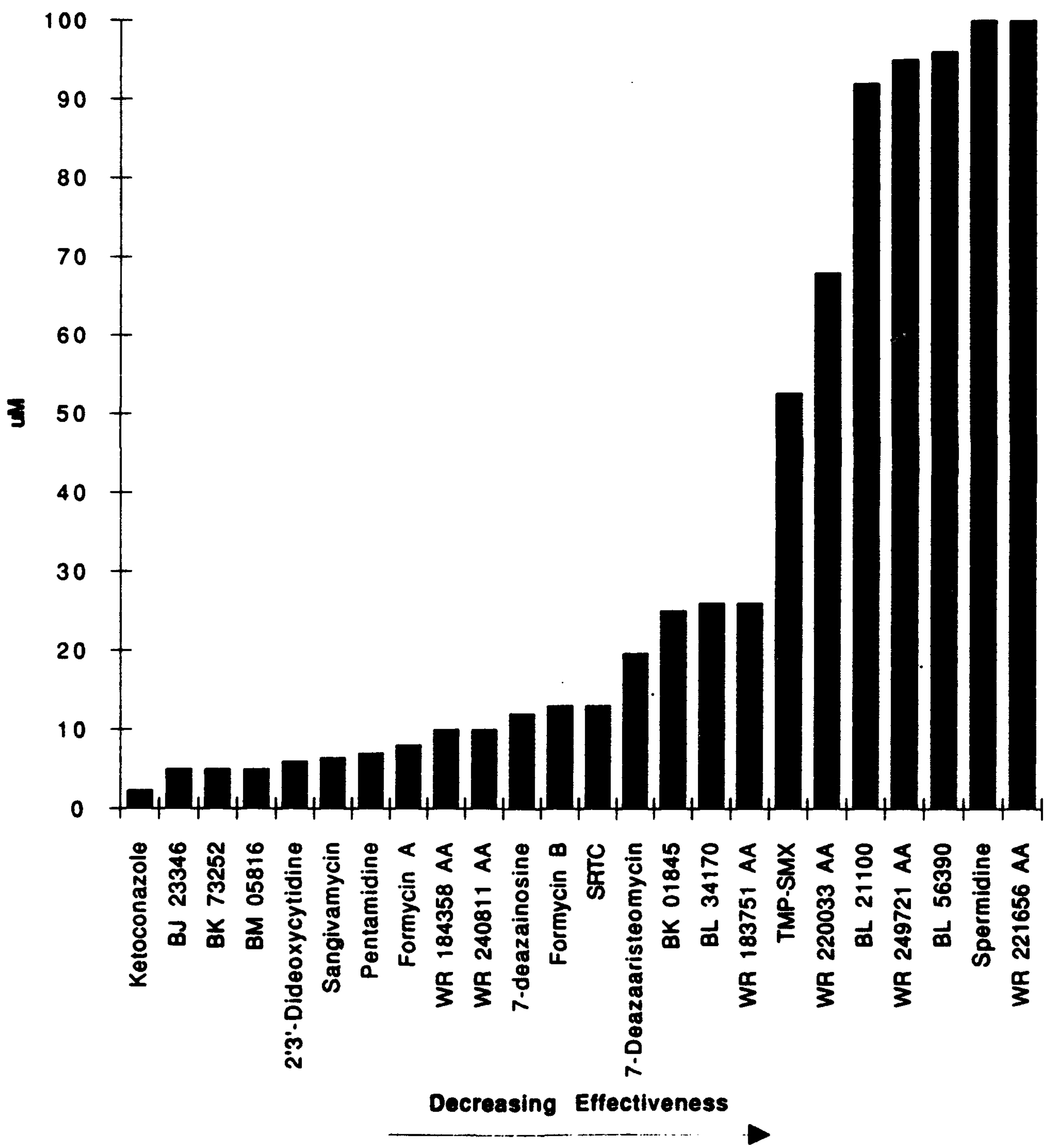
				A Polymerase			B Polymerase			
				Active	Inactive Compounds		Active	Inactive Compounds		
Bottle No.	WR No.	Name	Approved for Human Use	IC50	Max. % Inhib.	Highest Conc. Tested(uM)	IC50	Max. % Inhib.	Highest Conc. Tested(uM)	Comments
		Ara CTP			0	500	> 500		500	
		Aphidicolin			6	400		2	400	
		Ethidium Bromide		< 6			29			
		N-Ethylmaleimide		< 1000			1000			A Enzyme inhibited 94% by 5mM NEM, B Enzyme inhibited 86% by 5mM NEM
		Acyclovir			0	100		0	100	
		AZT			0	100		0	500	
	WR 2446				0	100		0	100	
	WR 783750				0	100		42	100	
		Pentamidine		100				NOT TESTED		
ZP 65105					33	500		0	500	
		dideoxy Inosine			21	500		NOT TESTED		
		dideoxy Cytosine			28	500		NOT TESTED		
		ddTTP			3	500	7.5			
		Spermine		250				0	500	
		Phosphomycin		1000				NOT TESTED		
		Azythromycin		500				NOT TESTED		
		Garlic Extract			17	1 (w/v)		NOT TESTED		
		Sinefungin			21	500		NOT TESTED		
		Oridion			30	200		NOT TESTED		
		Ponicidin			26	200		NOT TESTED		
		Cytosine 5' Carboxilic Acid			0	1000		NOT TESTED		
		Dapsone			12	33		NOT TESTED		
		Ketoconazole			11	10		NOT TESTED		
		Mitomycin C			11	2		NOT TESTED		
		Nalidixic Acid			15	10		NOT TESTED		
		Novobiocin			18	20		NOT TESTED		
		Sulfamethoxale			10	20		NOT TESTED		
		Trimethoprim			12	500		NOT TESTED		
		DFMO			0	2500		NOT TESTED		
		Berenil		40			50			
		BuPd GTP		25			5.4			
		BuAd ATP		150			50			
		Hemin		50			30			
		Suramin		7			2			
		Phosphonoacetic Acid		1500				0	2000	
		FPAA		130				0	160	
		BrPAA			0	800		0	800	
		ClPAA			0	800		0	800	
		F2PAA			10	2000		0	2000	

Bottle No.	WR No.	Name	Approved for Human Use		A Polymerase Active IC50	A Polymerase Inactive Compounds Max. % Inhib.	A Polymerase Inactive Compounds Highest Conc. Tested(uM)		B Polymerase Active IC50	B Polymerase Inactive Compounds Max. % Inhib.	B Polymerase Inactive Compounds Highest Conc. Tested(uM)	Comments
		COMDP				150	1000			0	1000	
		Arachidonic Acid			23-50*				40			*dependent on units of enzyme present
		Linoleic Acid			40				50			
		gamma-Linolenic Acid			40				40			
		Eicosapentaenoic Acid			140				207			
		Docohexaenoic Acid			150				192			

COMPOUNDS TESTED AGAINST HUMAN CEM T4 CELLS
(IC_{50} uM)



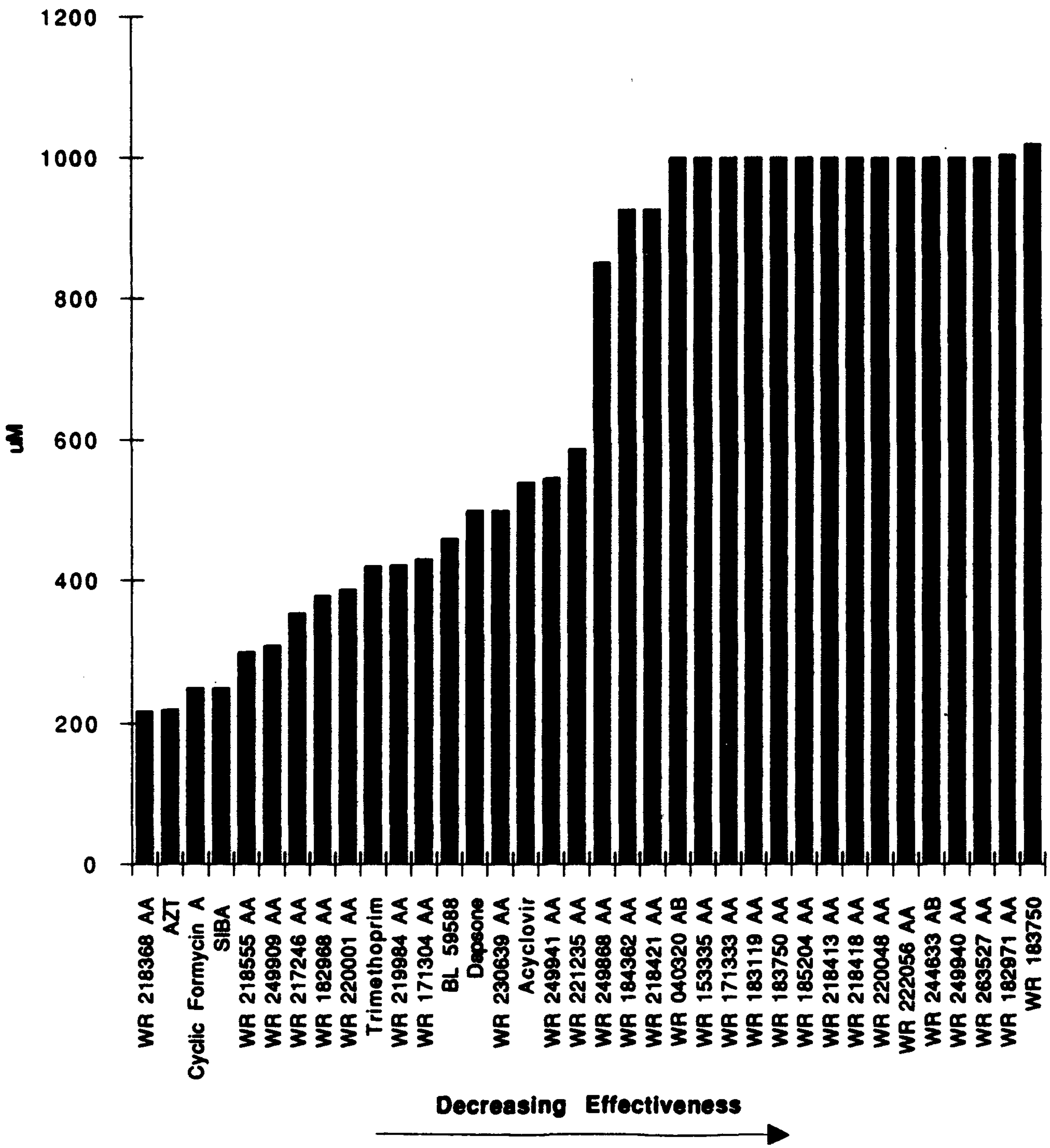
Very Effective Compounds for T4 Cells (IC50, uM)



Very Effective Compounds for T4 Cells (IC50, uM)

Compound	IC50, uM
Ketoconazole	2.3
BJ 23346	5
BK 73252	5
BM 05816	5
2'3'-Dideoxycytidine	6
Sangivamycin	6.4
Pentamidine	7
Formycin A	8
WR 184358 AA	10
WR 240811 AA	10
7-deazainosine	12
Formycin B	13
SRTC	13
7-Deazaaristeomycin	19.6
BK 01845	25
BL 34170	26
WR 183751 AA	26
TMP-SMX	52.6
WR 220033 AA	68
BL 21100	92
WR 249721 AA	95
BL 56390	96
Spermidine	100
WR 221656 AA	100

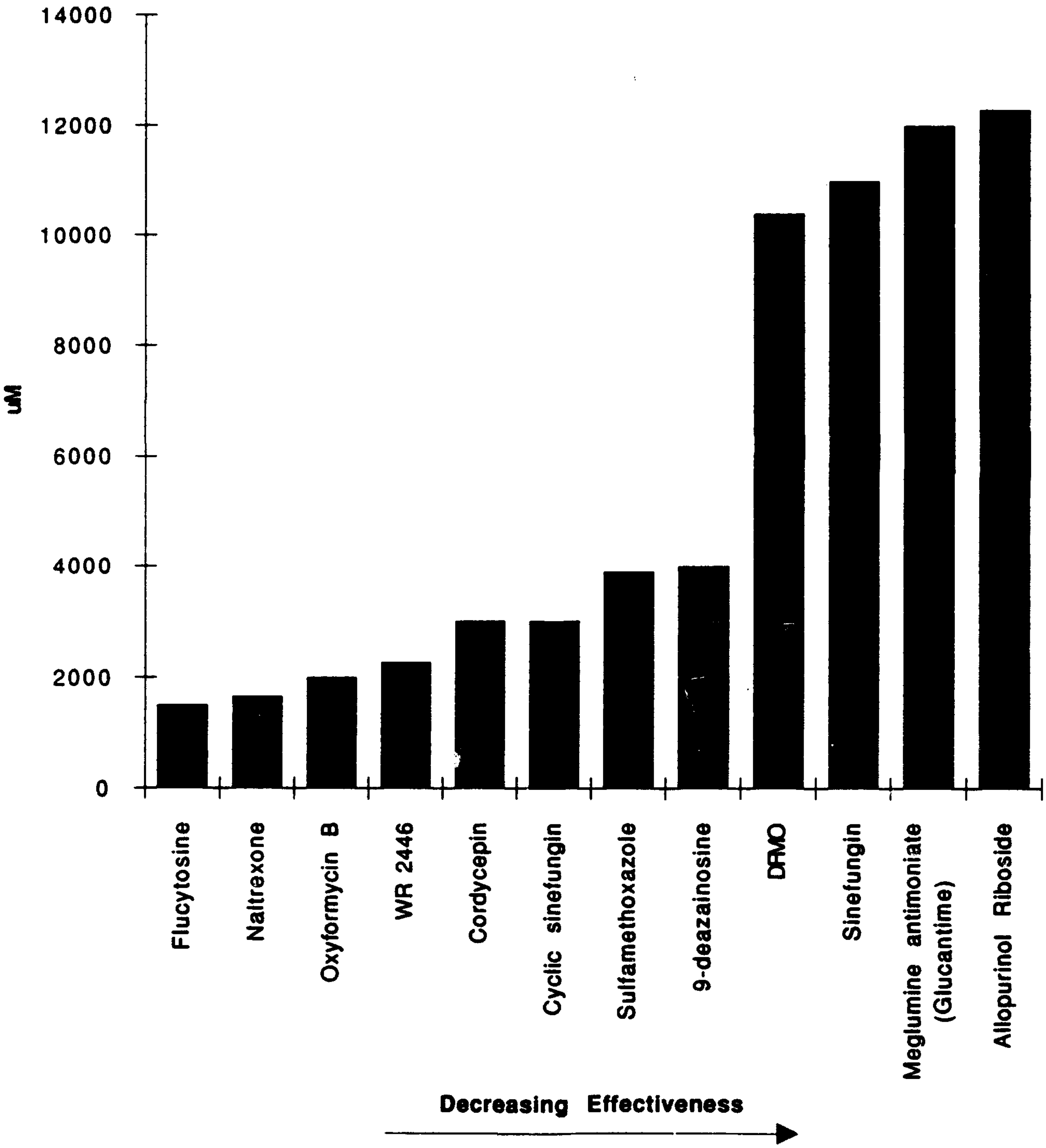
Moderately Effective Compunds for T4 Cells (IC50, uM)



Moderately Effective Compounds for T4 Cells (IC50, uM)

Compound	IC50, uM
WR 218368 AA	218
AZT	220
Cyclic Formycin A	250
SIBA	250
WR 218555 AA	300
WR 249909 AA	309
WR 217246 AA	354
WR 182968 AA	379
WR 220001 AA	388
Trimethoprim	420
WR 219984 AA	422
WR 171304 AA	430
BL 59588	460
Dapsone	500
WR 230639 AA	500
Acyclovir	540
WR 249941 AA	546
WR 221235 AA	587
WR 249868 AA	851
WR 184362 AA	927
WR 218421 AA	927
WR 040320 AB	1000
WR 153335 AA	1000
WR 171333 AA	1000
WR 183119 AA	1000
WR 183750 AA	1000
WR 185204 AA	1000
WR 218413 AA	1000
WR 218418 AA	1000
WR 220048 AA	1000
WR 222056 AA	1000
WR 244633 AB	1000
WR 249940 AA	1000
WR 263527 AA	1000
WR 182971 AA	1004
WR 183750	1020

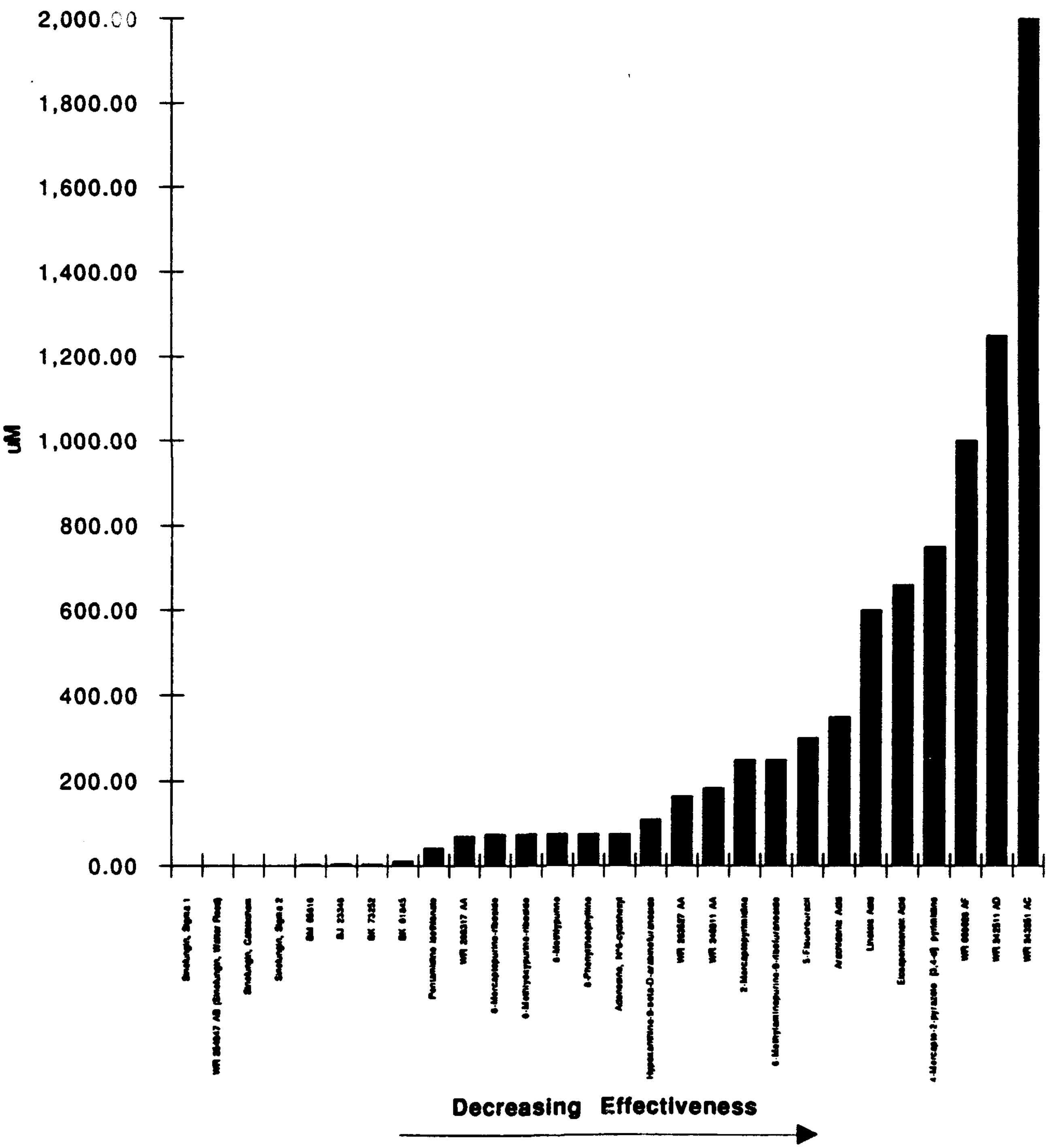
Least Effective Compounds for T4 Cells (IC50, uM)



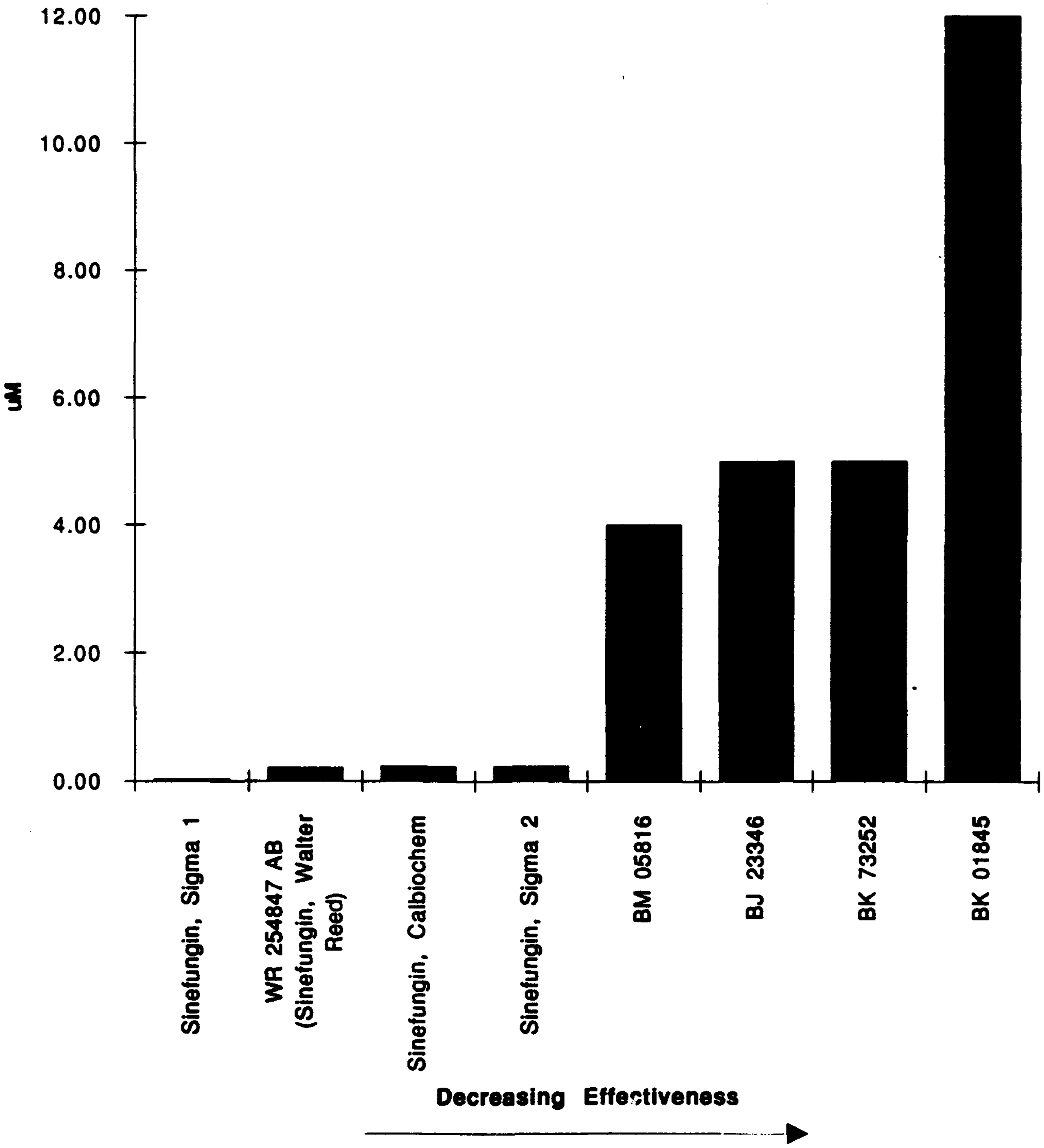
Least Effective Compounds for T4 Cells (IC50, uM)

Compound	IC50, uM
Flucytosine	1500
Naltrexone	1656
Oxyformycin B	2000
WR 2446	2270
Cordycepin	3000
Cyclic sinefungin	3000
Sulfamethoxazole	3900
9-deazainosine	4000
DFMO	10400
Sinefungin	11000
Meglumine antimoniate (Glucantime)	12000
Allopurinol Riboside	12300

Compounds for L. Mexicana (IC50, uM)



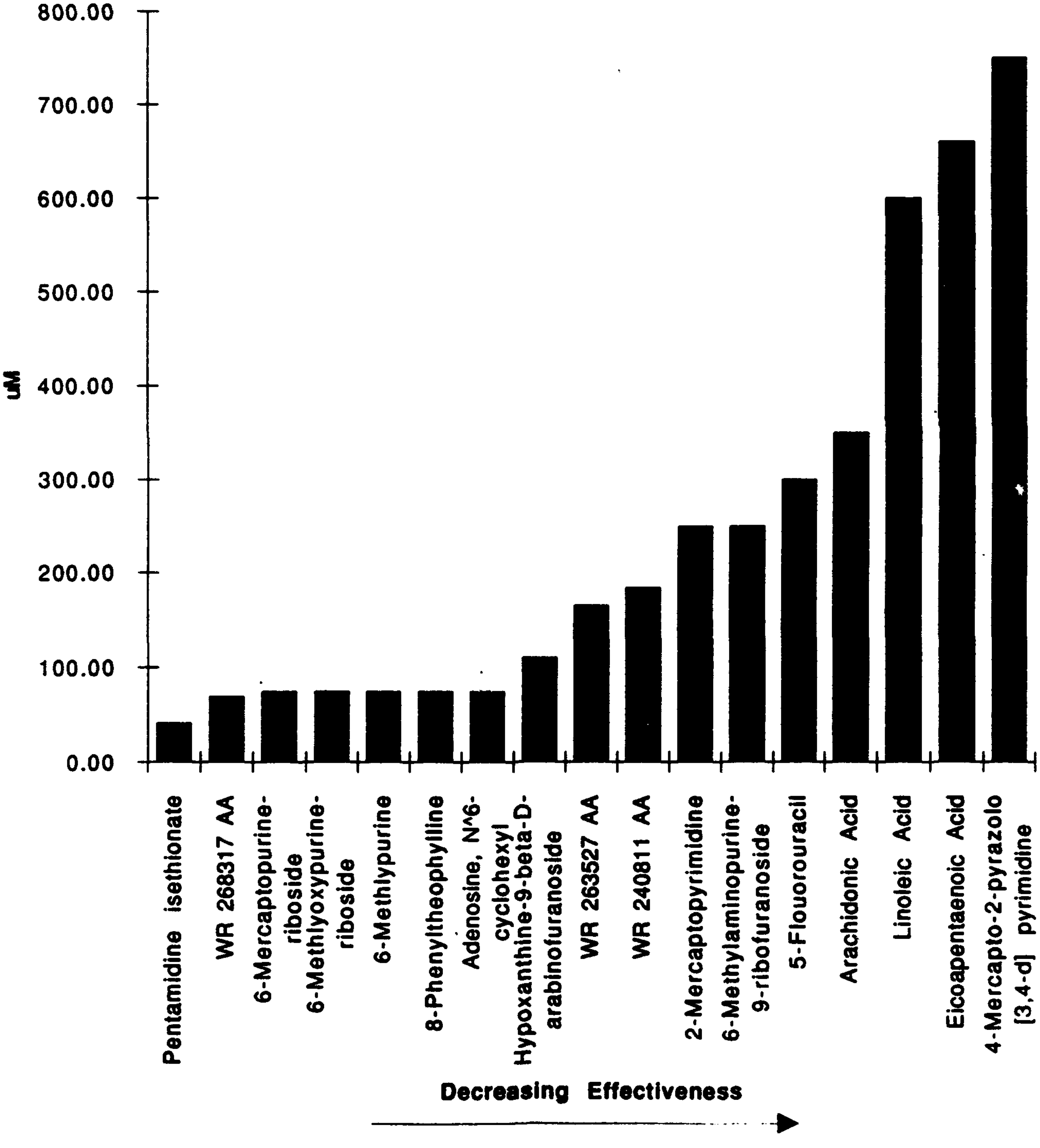
Very Effective Compounds for L. Mexicana (IC50, uM)



Very Effective Compounds for L. Mexicana

Compound	IC50, uM
Sinefungin, Sigma 1	0.03
WR 254847 AB (Sinefungin, Walter Reed)	0.21
Sinefungin, Calbiochem	0.24
Sinefungin, Sigma 2	0.24
BM 05816	4.00
BJ 23346	5.00
BK 73252	5.00
BK 01845	12.00

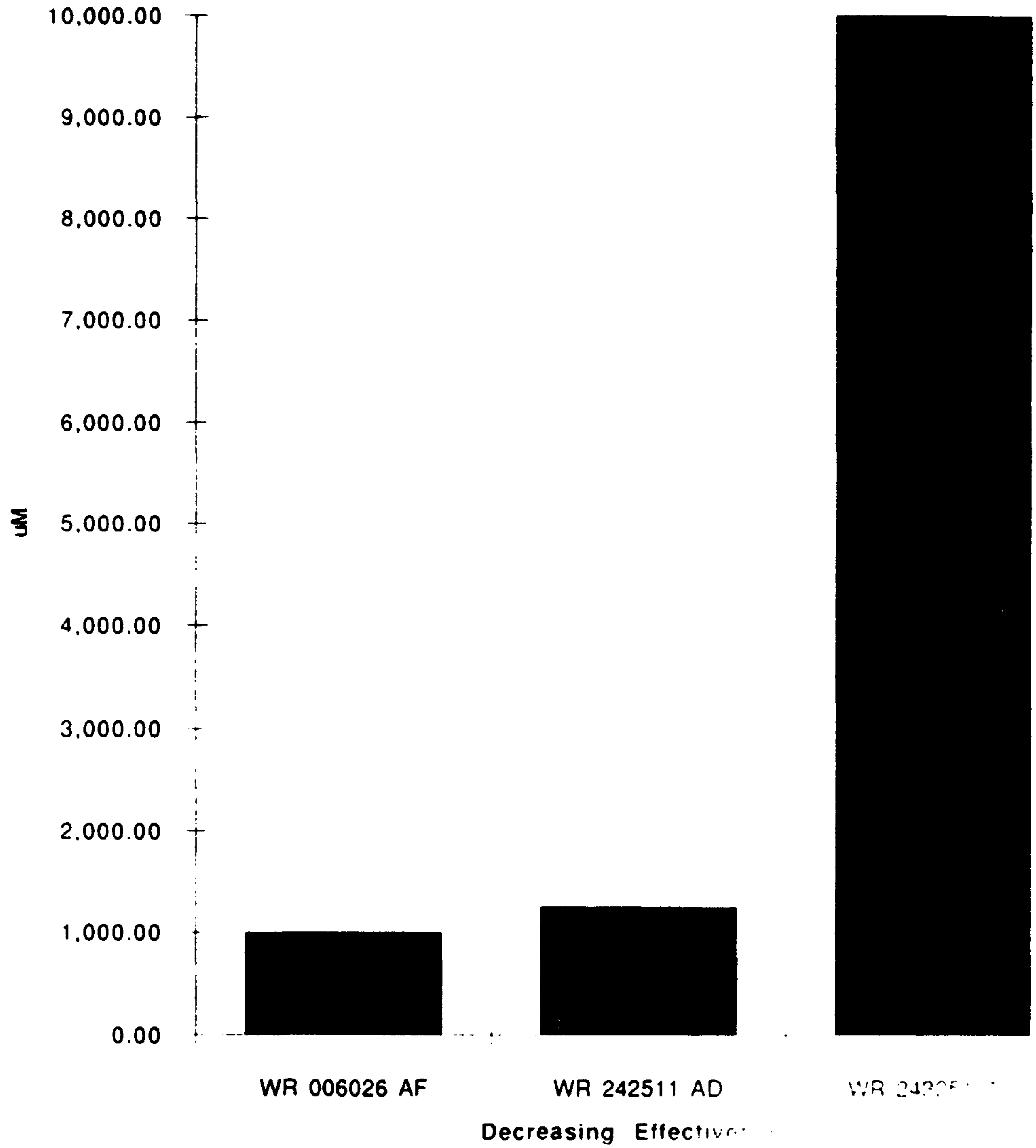
Moderately Effective Compounds for L. Mexicana (IC50, uM)



Moderately Effective Compounds for L. Mexicana

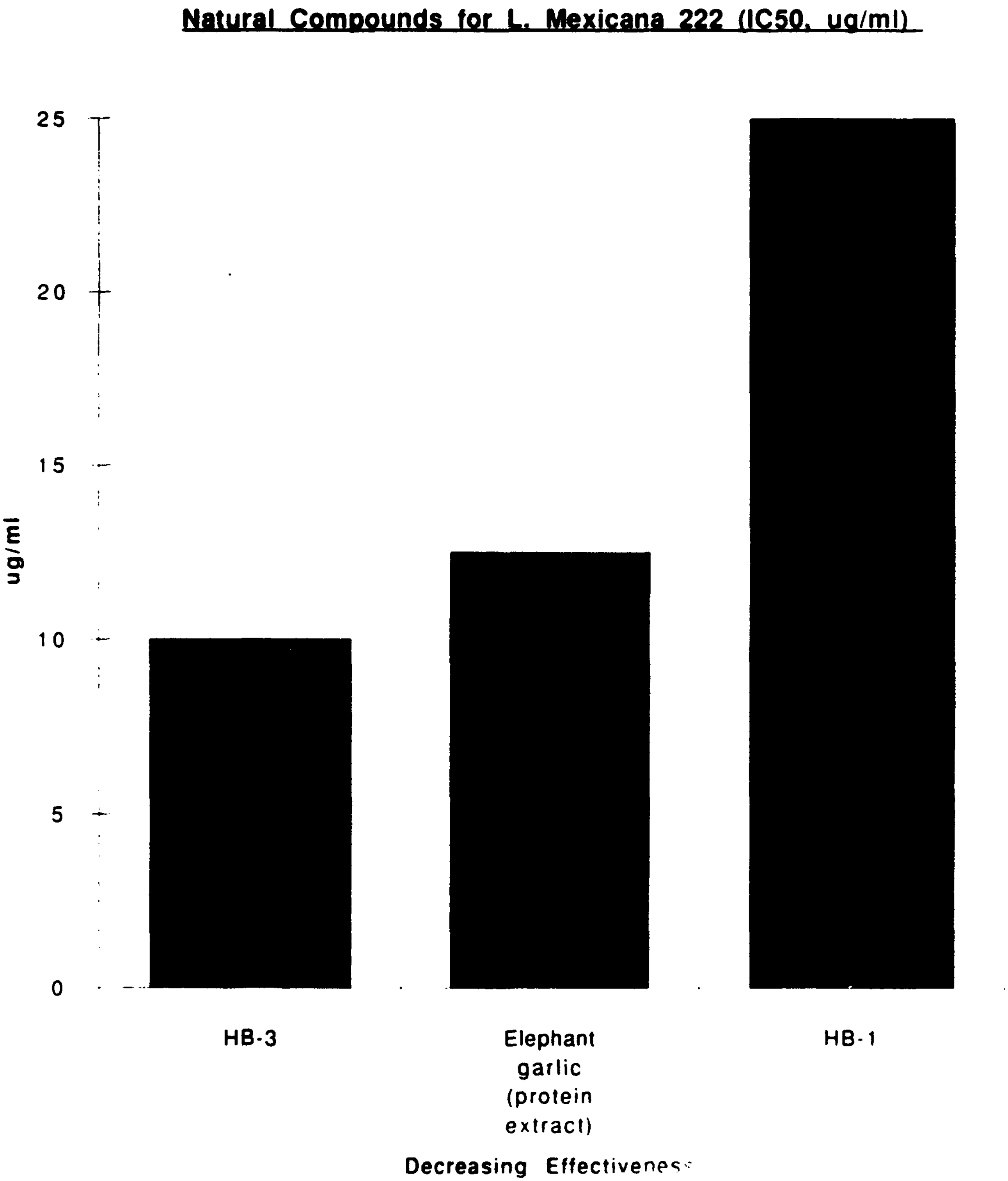
Compound	IC50, uM
Pentamidine isethionate	42.00
WR 268317 AA	70.00
6-Mercaptopurine-riboside	75.00
6-Methlyoxypurine-riboside	75.00
6-Methlypurine	75.00
8-Phenyltheophylline	75.00
Adenosine, N^6-cyclohexyl	75.00
Hypoxanthine-9-beta-D-arabinofuranoside	110.00
WR 263527 AA	165.00
WR 240811 AA	184.00
2-Mercaptopyrimidine	250.00
6-Methylaminopurine-9-ribofuranoside	250.00
5-Flouorouracil	300.00
Arachidonic Acid	350.00
Linoleic Acid	600.00
Eicoapentaenoic Acid	660.00
4-Mercapto-2-pyrazolo [3,4-d] pyrimidine	750.00

Least Effective Compounds for L. Mexicana (IC50, uM)



Least Effective Compounds for L. Mexicana

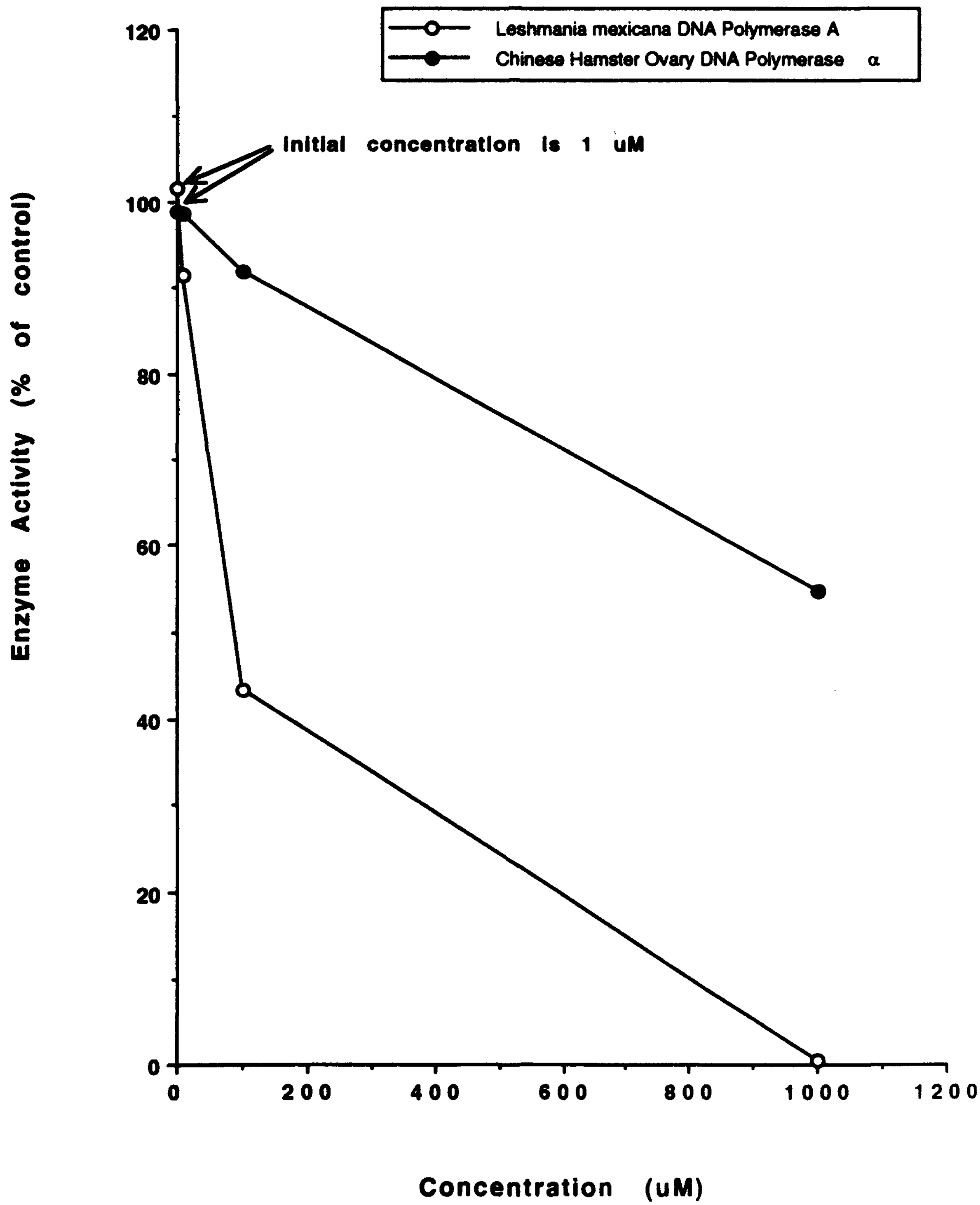
Compound	IC50, uM
WR 006026 AF	1,000.00
WR 242511 AD	1,250.00
WR 243251 AC	178,000.00


Natural Compounds for L. Mexicana 222 (IC50, ug/ml)
25
20
15
10
5
0
ug/ml
HB-3
Elephant garlic (protein extract)
HB-1
Decreasing Effectiveness

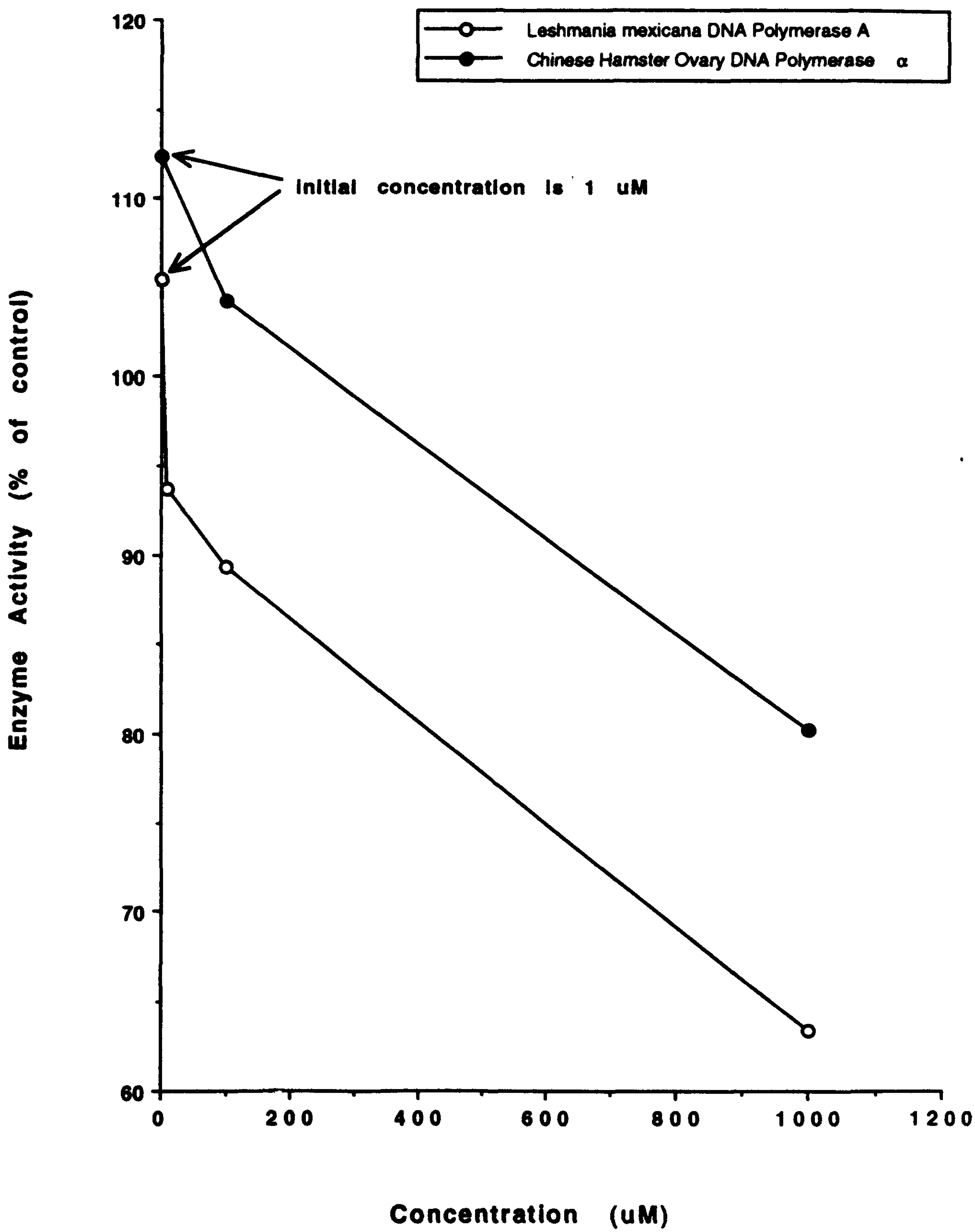
Natural Compounds for L. Mexiçana 222

Compound	IC50, ug/ml
HB-3	10.00
Elephant garlic (protein extract)	12.50
HB-1	25.00

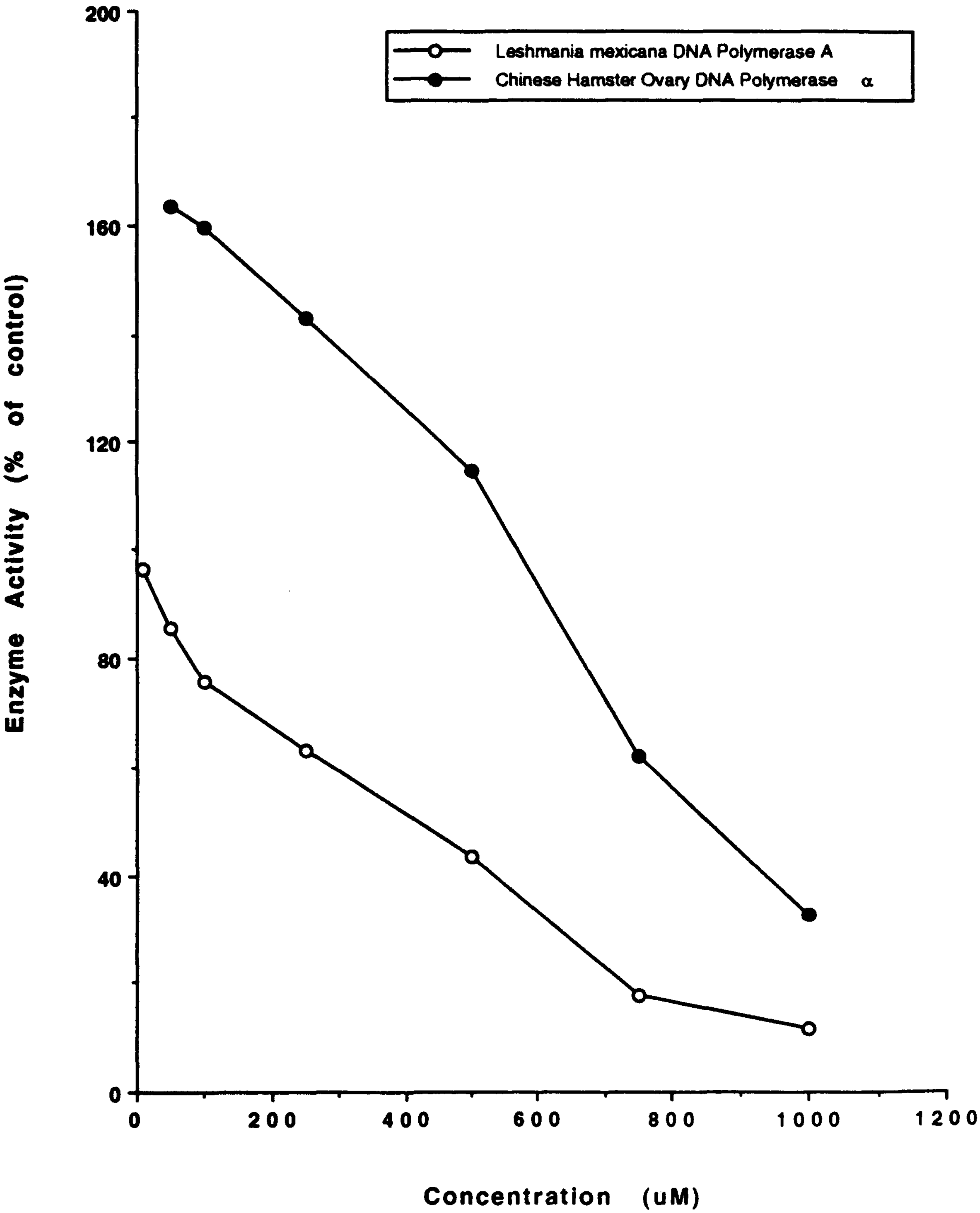
WRAIR Compound BJ 23346 Against Leshmania mexicana DNA Polymerase A and Chinese Hamster Ovary DNA Polymerase



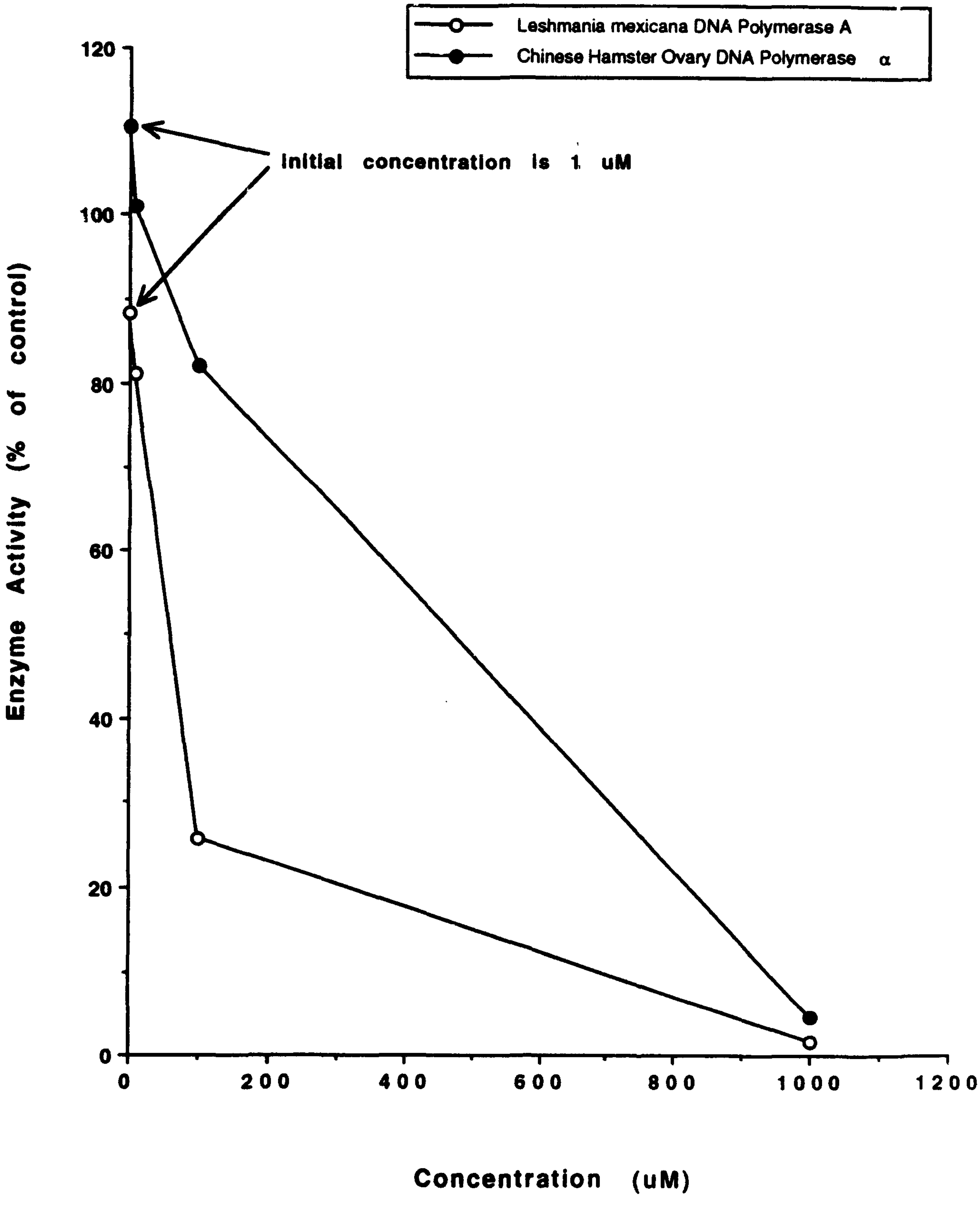
WRAIR Compound BK 01845 Against Leshmania mexicana DNA Polymerase A and Chinese Hamster Ovary DNA Polymerase



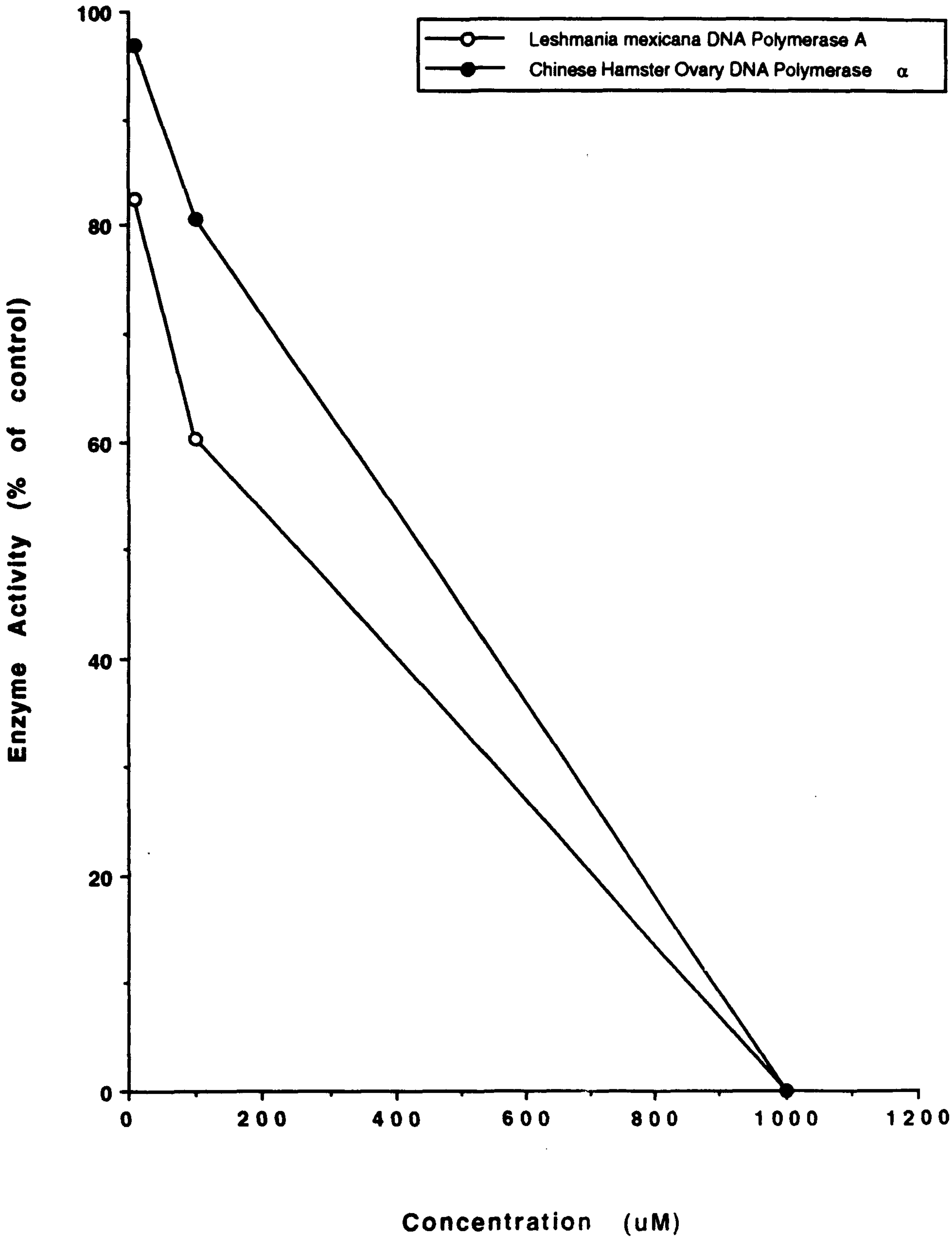
WRAIR Compound BK 40735 Against Leshmania mexicana DNA Polymerase A and Chinese Hamster Ovary DNA Polymerase



WRAIR Compound BK 73252 Against Leshmania mexicana DNA Polymerase A and Chinese Hamster Ovary DNA Polymerase



WRAIR Compound BM 05816 Against Leshmania mexicana DNA Polymerase A and Chinese Hamster Ovary DNA Polymerase



% Inhibition of Herbal Compounds on *Leishmania chagasi*

	% Inhibition	
COMPOUND	Concentration	
	0.17ug/ml	0.83 ug/ml
BM 12884	1.92	31.03
BM 12857	20.73	48.66
BM 12820	25.64	52.01
BM 12937	10.68	29.02
BM 12713	5.34	31.25
BM 12848	17.09	78.57
BM 12866	47.44	69.20
BM 12777	14.96	32.59

	Concentration
	75ug/ml
BM 12946	Stimulation
	% Inhibition
BM 12759	28.37
BM 12722	26.20
BM 12811	58.65
BM 12875	68.03
BM 12768	23.56
BM 12802	75.24
BM 12919	86.54

Effect of Natural Compounds on Leishmani chagasi and Human CEMT4 cells

Cells	Compound	Concentration - ug/ml	% Inhibition
Leishmania chagasi	PS-55	1-15	None
	PS-55	25	3.1
	PS-55	50	14.2
	DML-55	1-25	None
		50	13.2
Human CEMT4	PS-55	1-25	None
	PS-55	50	48
	DML-55	1-15	None
	DML-55	25	19
	DML-55	50	35

THE DNA POLYMERASES OF *LEISHMANIA MEXICANA*

SUMMARY

Two previously isolated DNA polymerases from the parasitic protozoan *Leishmania mexicana* were further characterized by exposure to inhibitors of mammalian DNA polymerases. DNA polymerase A, a high molecular weight enzyme, and DNA polymerase B, a β-like DNA polymerase were compared to each other and to their mammalian counterparts regarding pH optimum, utilization of templates, and response to various inhibitors and ionic strengths. The results suggest the DNA polymerases from *L. mexicana* differ from the host enzymes and may offer a target for chemotherapeutic intervention.

INTRODUCTION

Five classes of DNA polymerase (α, β, γ, δ and ε) have been isolated from higher eukaryotic cells (5-9) and α, β and γ-like polymerases from parasitic protozoa (1-18). DNA polymerases α, δ and ε are nuclear enzymes associated with chromosomal replication; β is a low molecular weight nuclear enzyme involved in DNA repair (2,15,16), and γ which has been isolated from mitochondria is believed to be responsible for mitochondrial DNA replication (6,9,21).

We have been studying DNA replication in the kinetoplast parasite *Leishmania mexicana* and have begun studies to characterize the major polymerase activities in these parasites for the purpose of comparing them to host polymerases, particularly α and β. Although North and Wyler reported studies of *in vivo* DNA replication of *Leishmania* parasites (22), this laboratory is the first to report the isolation and characterization of the leishmanial DNA polymerases in vitro (10-12). Others have described purification of α-like, β-like (13) and γ-like polymerases (18) from the parasitic protozoans *Crithidia fasciculata* and an α-like polymerase from *Trypanosoma* brucei (14) and *Trypanosoma cruzi* (15).

The purpose of this study is to compare the major DNA polymerase activities (A and B) isolated from *Leishmania mexicana* to α and β polymerases isolated from other sources.

MATERIALS AND METHODS

Test organism

Leishmania mexicana amazonensis (Walter Reed strain 227) obtained from the Leishmania section of the Walter Reed Army Institute of Research were grown in brain heart infusion media as previously described (10).

Preparation of Compounds

Aphidicolin (Sigma Chemical Co., St. Louis, MO) was prepared in dimethylsulfoxide (DMSO) as a 5 mM stock and diluted with water so that the final concentration of DMSO in the assay was no more than 0.16% (v/v). Suramin, purchased from Miles Pharmaceuticals (West Haven, CT), was made into a 70 mM stock solution in 10 mM Tris pH 7.5. Further dilutions were made with the same buffer. Butylphenyl dGTP (BuPdGTP), carbonyldiphosphonate (COMDP) and the phonoacetic acid derivatives BrPAA, ClPAA, FPAA, and F_2PAA were generous gifts from Dr. Wright, University of Massachusetts Medical Center (Worcester, MA), and were prepared in aqueous solution at the appropriate concentrations. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of DNA polymerases

DNA polymerase A, a high molecular weight DNA polymerase sensitive to N-ethylmaleimide (NEM) was isolated from *L. mexicana* promastigotes as described (11). A low molecular weight DNA polymerase classified as a β-like enzyme was isolated from promastigotes as described (12) This enzyme will be referred to as DNA polymerase B, to distinguish it from mammalian enzymes and to follow the designation used by Holmes et al. (13) for the *Crithidia fasciculata* DNA polymerases.

Drug assays

The inhibitory properties of several compounds were determined by pre-incubating the enzyme and drug in the assay mix. In order to characterize the enzymes, selective inhibitors of mammalian DNA polymerases were tested against both enzymes. DNA polymerase A was assayed at 35°C as described (11). DNA polymerase B activity was measured at 35°C as previously described (12).

RESULTS AND DISCUSSION

Isolation of DNA polymerases

Two types of DNA polymerase activity were separated using affinity chromatography with denatured DNA cellulose. The two enzyme activities were designated as DNA polymerase A (11) and a β-like DNA polymerase (12) according to their molecular weight, pH optimum and response to N-ethylmaleimide.

The B enzyme (pol B) was less stable than the DNA polymerase A (pol A) at all stages of the purification. The use of a mixture of protease inhibitors as well as glycerol during the isolation procedures was essential for stability of the DNA polymerases. In addition, the use of

a freshly prepared assay mix was critical in obtaining pol B enzyme activity. Difficulty in detecting a low molecular weight DNA polymerase in parasitic protozoans has resulted in conflicting reports from some groups regarding the presence of a low molecular weight DNA polymerase in *Trypanosoma brucei* (9, 14). In addition, studies of *T. cruzi* detected only one DNA polymerase of high MW and no β-like enzymes (15).

Proper characterization of the DNA polymerases from *L. mexicana* is essential in order to compare them with the host enzymes, a first step in a strategy to develop chemotherapeutic agents. To date, all enzymes isolated from parasitic protozoans have been found to share some, but not all, of the characteristics of the mammalian enzymes (9, 11-18).

Characterization Studies

Pol A was slightly stimulated by NaCl or KCl at concentrations of less that 15mM, but rapidly inactivated by higher concentrations of salt (11). DNA polymerase B was slightly stimulated by 5 mM KCl only, but was more resistant to inactivation by higher concentrations of NaCl or KCl, with 35% of the activity remaining in the presence of 200 mM NaCl and 43% of the activity remaining in the presence of 200 mM KCl (12). Mammalian DNA polymerase α is inhibited by high ($\geq$ 100 mM) concentrations of salt, whereas DNA polymerases β and δ are stimulated by such concentrations (6).

The optimum pH of the pol A enzyme is mildly acidic to neutral at 6.7, whereas the optimum pH of the pol B enzyme is basic, at 9.0. Table 1 shows a comparison of the activity of the enzymes with several template-primers. The pol A had a template preference for activated DNA and used poly (dA) · oligo (dT) $_{12-18}$ equally as well, with only 60% of the activity when poly (dC) · oligo(dT) $_{12-18}$ was the template (11). Pol B showed a six-fold preference for poly (dC) · oligo(dT) $_{12-18}$ as the template over activated DNA (8). The preferred template for DNA polymerases α and β is activated DNA, whereas the mitochondrial DNA polymerase is more active with poly (rA) · Oligo (dT). A notable point is the inability of pol B to utilize Mn^{+2} as the divalent cation activator. In contrast, mammalian DNA polymerase β is capable of using both Mn^{+2} and Mg^{+2} (6).

Inhibitor Studies

Exposure of the enzymes to specific DNA polymerase inhibitors showed the *L. mexicana* enzymes to be different from one another and from mammalian enzymes in their sensitivity to various compounds (Table 2). Both enzymes were resistant to aphidicolin, a mammalian DNA polymerases α, δ and ε inhibitor. The response of these enzymes to the mammalian DNA polymerase α inhibitor BuPdGTP was interesting. In the presence of 100 1M dGTP, Pol B was twenty fold more sensitive to this compound than Pol A with a concentration that inhibits activity by 50% (IC_{50}) of 5.4 1M, whereas the Pol A was inhibited with an IC_{50} of 100 1M.

Phosphonoacetic acid (PAA) was a weak inhibitor of the Pol A with only 35% inhibition at 2 mM. Pol B was resistant to PAA at concentrations of up to 2 mM. Mammalian β polymerase has been found to be resistant to inhibition by this compound (6). Several PAA analogues (25) were tested against the L. *mexicana* DNA polymerases (Table 2). Pol B was completely resistant to inhibition by the fluoro, bromo, chloro, and difluoro analogues of PAA (FPAA, BrPAA, C1PAA, F_2PAA; respectively). Pol A was resistant to BrPAA, C1PAA, and F_2PAA. FPAA, a monohalogenated derivative of PAA, inhibited pol A with an IC_{50} of 130 1M, resulting in over a ten fold increase in inhibition compared to PAA. FPAA also exhibited potent inhibition of the calf thymus DNA polymerases α and δ [Table 2; (25)]. COMDP, a specific inhibitor of mammalian DNA polymerase δ (19, 26) and Dr. G. Wright, personal communication), was inhibitory to both enzymes from *L. mexicana*. The Pol A enzyme was more sensitive to COMDP than the pol B with IC_{50}'s of 150 and 200 1M, respectively (Table 2).

The response of these *L. mexicana* enzymes to non-specific inhibitors showed the unique properties of each enzyme (Table 2). Hemin, a critical nutritional component of the leishmanial growth media (10) was found to inhibit both enzymes, inhibiting pol B with an IC_{50} of 60 1M versus an IC_{50} of 90 1M for the pol A. Hemin inhibits DNA synthesis reversibly by binding DNA polymerase and causing it to dissociate from the template (27). Suramin, a drug used in the treatment of trypanosomiasis that has also been found to be a strong competitive inhibitor of the reverse transcriptase of a number of animal retroviruses (28), was found to be a potent inhibitor of the *L. mexicana* DNA polymerases. Suramin gave an IC_{50} of 8 1M, for pol A and 3 1M, for pol B (Table 2).

Our characterization studies have shown the *L. mexicana* pol A and pol B to differ from each other in molecular weight, pH optimum, template specificity, and response to salt and inhibitors. In addition, our studies have shown that pol A and pol B share similar properties such as pH optimum, molecular weight, and sensitivity to specific inhibitors such as NEM with their mammalian counterparts. The assignment of pol A to a specific class among the eukaryotic DNA polymerases is made difficult by its utilization of template (Table 1) and by the particular response of this enzyme to inhibitors (Table 2). Although this high molecular weight enzyme shows α-like properties such an inhibition by NEM and salt, insensitivity to ddTTP, and preference for Mg^{+2} and activated DNA, it also displays characteristics that do not fit the type. Pol A also shows characteristics of the δ type, such as resistance to aphidicolin and utilization (although at low levels, Table 1) of ribonucleotide template when Mn^{+2} is the divalent cation. On the other hand, the low sensitivity to BuPdGTP and the somewhat high sensitivity to COMDP point toward characteristics of the DNA polymerases δ and ε (24).

Pol B can be more easily classified as β-like enzyme based on its low molecular weight, resistance to NEM, and sensitivity to ddTTP. However, Pol B failed to crossreact with an anti-

recombinant mouse DNA polymerase β antiserum enzyme neutralization studies (12). Using enzyme neutralization studies, as well as immunodiffusion and immunoelectrophoresis, Chang and Bollum showed that *T. brucei* DNA polymerase β did not crossreact with an antiserum against calf thymus DNA polymerase β (29).

Observations of differences with the mammalian polymerase have been made on the enzyme of other protozoans (11-18, 24,29) suggesting that DNA replication in higher eukaryotes and protozoans may differ. Such differences are being characterized in this laboratory in the search for potential ant. parasitic agents.

PARTIAL PURIFICATION AND CHARACTERIZATION OF THE ISOZYMES OF S-ADENOSYLMETHIONINE SYNTHETASE FROM *LEISHMANIA MEXICANA*

SUMMARY: Two forms of AdoMet synthetase were separated from the parasitic protozoan *Leishmania mexicana*. The purification procedures involved ammonium sulfate fractionation, DEAE-cellulose chromatography and Sephacryl S-200 HR gel filtration resulting in a 2483 fold purification for the α isozyme and 2417 fold for the β isozyme. The α and β isoforms follow Michaelis-Menten kinetics with apparent Km values for methionine of 357 μM for α and 270 μM for β. The apparent molecular weights were determined to be 91 kDa for α-isozyme and 44 kDa for β-isozyme. Markedly different molecular weights have been reported from mammalian sources. The AdoMet synthetase α and β isozymes differed in pH optimum, thermal stability, isoelectric points, and response to metal ions and various inhibitors. This is the first report on the isolation of AdoMet synthetase from *Leishmania sp.*

INTRODUCTION

S-Adenosylmethionine (AdoMet) Synthetase [ATP:L-methionine S-adenosyl transferase, EC 2.5.1.6] catalyzes the formation of S-adenosyl-L-methionine (AdoMet). AdoMet is a naturally occurring molecule distributed in all biological tissues and fluids (1). It is of fundamental importance in a number of biological reactions involving enzymatic transmethylation, contributing to the synthesis, activation and/or metabolism of such compounds as hormones, neurotransmitters, nucleic acids, proteins, phospholipids and carbohydrates (2). AdoMet is also a precursor to the polyamines. The naturally occurring polyamines putrescine, spermidine, and spermine are organic cations widely distributed in both prokaryotic and eukaryotic organisms. Polyamine synthesis increases and polyamine levels rise when the growth rate is maximal. Growth appears to be related to and dependent upon polyamine biosynthesis (3).

Since our previous studies have indicated that one of the most potent antileishmanial agents to date, sinefungin, an AdoMet analogue interferes indirectly with nucleic acid metabolism (4), we have begun studies to determine the significance and uniqueness of the parasitic Sadenosylmethionine synthetase (the enzyme which produces AdoMet).

The essentiality of AdoMet in transmethylation reactions and polyamine biosynthesis provides a rationale for the development of antileishmanial methionine analogues. To our knowledge, no reports have occurred on the AdoMet synthetase of the lower eukaryotic parasitic protozoan *Leishmania mexicana*. It is hoped that elucidation of key differences between parasite and host enzymes or the requirement of the enzyme product for parasite survival will offer a target for chemotherapeutic exploitation.

MATERIALS AND METHODS

Materials

L-cis-AMB (L-2-amino-4-methoxy-cis-but-3-enoic acid) was synthesized by Sufrin et al. with minor modifications in the published procedure (5). Methapyrilene hydrochloride was a gift from Dr. William Lijinsky, NCI- Frederick Cancer Research Facility, BRI - Basic Research Program, Frederick, MD. L-[methyl- ^{3}H] methionine (83.2 Ci/m mol) was purchased from Amersham Corp. (Arlington Heights, Ill.) Fisher ScintiVerse liquid counting cocktail and enzyme grade ammonium sulfate were obtained from Fisher Scientific (Fair Lawn, NJ). DEAE-cellulose (DE 23, Fibrous) and P81 cellulose phosphate cation-exchange paper discs were from Whatman (Clifton, NJ). Sephacryl S-200 HR was from Pharmacia LKB Biotechnology (Piscataway, NJ). Centricon-10 and Centriprep-10 concentrators were from Amicon (Danvers, MA). Molecular weight standards for gel filtration, IEF proteins standard, Bio-Lyte 8-10 and Coomassie Brilliant Blue R-250 were from Bio Rad Laboratories (Richmond, CA). Resolyte 3-10 was from Hoefer Scientific Instruments, Electran BDH Chemicals Ltd. (Poole, England). IsoGel IEF grade agarose was from FMC Corp. (Rockland, ME). 5-Azacytidine was from Fluka Chemical Corp. (Ronkonkoma, NY). All other reagents and laboratory chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

Cell culture for enzyme isolations

Promastigotes of *Leishmania mexicana amazonensis* (Walter Reed strain 227) was originally obtained from the Leishmania Section of the Walter Reed Army Institute of Research, Washington, D.C. Promastigotes were grown as previously described (4).

AdoMet synthetase assay

The activity of AdoMet synthetase was determined according to the method of Hoffman and Kunz (6) with a slight modification to optimize our enzyme assay. The standard reaction mixture in a total volume of 100 µl contained 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 150 mM KCl, 5 mM DTT, 10 mM ATP, 10 µM L-[methyl- ^{3}H]methionine, and 36 µl enzyme. The incubation was carried out at 35°C for 20 min. The reaction was terminated by cooling in an ice bath. Seventy-five µl portions of the reaction mixtures were spotted on 2.3 cm discs of Whatman P81 cellulose phosphate cation-exchange paper, dried with a hair dryer, and washed 5 times, with 10 ml/filter, in cold 0.1 M ammonium formate (pH 3.0) to remove the unreacted labelled methionine. The washed filters were then treated two times with 95% ethanol to remove water, and once with ethyl ether to remove the alcohol. [^{3}H]-AdoMet was quantified by liquid scintillation counting of dried filters under 5 ml of Fisher ScintiVerse. One unit of the enzyme

was defined as the amount of enzyme that catalyzes the formation of 1 pmol of AdoMet/min at 35°C. Unless otherwise noted, all assays were performed in duplicate.

Protein determination

Protein concentrations were estimated according to the modified dye binding method of Redinbaugh and Campbell (7) using bovine serum albumin as the standard.

Isolation of two AdoMet synthetase isozymes from L. mexicana

All operations were carried out at 0-4°C. *Leishmania mexicana* 227 promastigotes were grown as previously described (4). The crude enzymes were isolated by suspending 12-18g of pelletted wet cells in approximately 15 ml buffer A containing 20 mM potassium phosphate pH 7.5, 0.1 mM EDTA, 1 mM DTT, 0.02% sodium azide. The cell suspension was then sonicated 3 times for 15 sec each at an output of 160 watts on a Braun-Sonic 2000 sonicator. A mixture of protease inhibitors were added immediately after sonication to a final concentration of 48 μg/ml trypsin inhibitor, 48 μg/ml aprotinin, 20 μg/ml leupeptin and 1 mM phenylmethyl-sulfonyl fluoride (PMSF). The broken cell suspensions (step 1) were centrifuged at 45,000 x g for 90 min at 4°C. The supernatant fluid was passed through a layer of glass wool to remove the lipid layer (step 2). The crude extract (21 ml) was then fractionated with ammonium sulfate (75% saturation). The precipitated proteins were dissolved in 13 ml of buffer A and then dialyzed at 4°C against 200 volumes of the same buffer (step 3). The dialyzed sample (18 ml) was loaded on a 1.5 x 13 cm DEAEcellulose column equilibrated with buffer A at a flow rate of 60 ml/hr. The column was washed with buffer A until 280 nm-absorbing material in the eluate was negligible. Enzyme was eluted using a 230 ml linear gradient of 0-0.4 M KCl in buffer A. Fractions of 2.5 ml were collected. A typical elution pattern of AdoMet synthetase is shown (Fig. 1).

The active fractions with activity of 50% of maximum or greater were retained and pooled (step 4). The pooled DEAE-cellulose eluate (75 ml) was brought to 75% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation (39,000 x g, 30 min) and redissolved in a minimal volume (3 ml) of buffer B containing 20 mM potassium phosphate pH 7.0, 0.2 M KCl, 1 mM DTT, 0.1 mM EDTA, 20% (v/v) glycerol, 0.02% sodium azide. The enzyme suspension was concentrated and dialyzed by Centricon-10 using buffer B, then chromatographed on a column (1.5 x 75 cm) of Sephacryl S-200 HR equilibrated with buffer B at a flow rate of 48 ml/hr (Fig. 2). Fractions of 1 ml were collected, and fraction numbers 58-62 (AdoMet synthetase α) and 7375 (AdoMet synthetase β) were pooled (step 5). The intermediate fractions were discarded to avoid possible cross-contamination. The pooled active fractions from

Sephacryl S-200 HR were concentrated by ultrafiltration using Centriprep-10 to about 1,500-2,500 units/ml and stored at -70°C for characterization studies.

Molecular weight determination by gel filtration chromatography

Gel filtration chromatography was carried out at 4°C on a column of Sephacryl S-200 HR (1.5 x 75 cm) equilibrated in buffer B by using a flow rate of 48 ml/hr. The apparent molecular weights of the two isozymes were estimated by using thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.35 kDa) as marker proteins.

pH optimum determination

Buffers with concentrations of 500 mM, ranging in pH from 7.2 to 10.2, were prepared (Tris buffer, pH 7.2-9.0; 2-amino-2-methyl-1,3-propane-diol, AMPD buffer, pH 9.0-10.0; (2-[N-cyclohexylamino]-ethane-sulfonic acid, CHES buffer, pH 9.6-10.2). Buffers used were substituted in the assay mixture at a final concentration of 50 mM.

Effect of divalent cations

Divalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+}, Fe^{2+}) were added to the assay mixture at final concentrations of 20 mM. All were in chloride form. The assays were performed in duplicate in a final volume of 100 μl as described above. Attempts to remove EDTA and KCl from the enzyme preparations by dialysis resulted in the loss of enzyme activity; therefore the enzymes (in buffer B) were used without dialysis before assays with various cations.

Thermal stability

Thermal stability was determined by subjecting the enzymes to different temperatures at various times before the assay procedure.

Isoelectric focussing

Analytical isoelectric focussing was performed using Hoefer Scientific SE 250 Mighty Small vertical gel unit according to manufacture's suggested protocol (Hoefer Scientific Instruments Technical Bulletin #134, Agarose Slab Gel IEF in the SE 250). The pH range of 3-10 was achieved with solutions of 1.8% Resolyte (3-10) and 0.7% of Bio-Lyte (8-10), using 0.02 M phosphoric acid and 0.02 M sodium hydroxide as the anode and cathode electrolytes, respectively. Sephacryl S-200 HR purified enzymes were concentrated by Centriprep-10 to 7,500-10,000 units/ml. Aliquots (10 μl) each of AdoMet synthetase α and β were separately loaded to

the agarose slab gel in addition to Bio-Rad IEF protein standards. Focussing was carried out at room temperature with tap water cooling at 200 volts for 30 min., then at 600 volts for an additional 1 hr. Two gel lanes (α and β) were sliced in 0.2-cm sections and extracted with 92 μl of standard assay mixture for 18 hr at 4°C, then assayed at 35°C for 20 min. The remaining gel was fixed and stained. Focused IEF standard protein bands were used as references for isoelectric points of AdoMet synthetase α and β.

Inhibitory Studies

Sephacryl S-200 HR purified and concentrated AdoMet synthetase α and β isoenzymes were assayed for inhibition of activities by various compounds. Each compound was tested by including it in the assay mixture (as previously described) at various concentrations. All compounds were made into stock solutions with water except dimethylsulfoxide.

RESULTS AND DISCUSSION

Two isoforms of AdoMet synthetase α and β have been isolated from *Leishmania mexicana*. The purification procedures, involving ammonium sulfate fractionation, DEAE-cellulose chromatography, and Sephacryl S-200 HR gel filtration resulted in 2483-fold and 2417-fold purification of AdoMet synthetase α and β, respectively. A total of 138% of AdoMet synthetase activities were recovered. Although two isozymes were purified only about 2000-fold, it was not the aim of this study to purify them to homogeneity but rather to separate two isoforms from each other in order to study their characteristics.

The activity of the enzyme was increased sharply after ammonium sulfate fractionation and DEAE-cellulose chromatography (Table 1), indicating the possibility that an endogenous AdoMet synthetase inhibitor might be removed at these steps or the substances that might interfere with the AdoMet synthetase assay were removed.

The partial purified isozymes were stable in buffer B for 6 days at -70°C, -20°C and 4°C (Table 2). When the isozymes were stored at -70°C for 4 months, the α enzyme lost 15% of activity, and the β enzyme lost 31%. Greater loss of activity was observed when both isozymes were stored at -20°C for 4 months. Heating to 45°C resulted in a loss of activity of 17% and 66% for the α and β enzymes, respectively. No activity remained for the α enzyme when heating at 75°C for 30 min., and for the β enzyme when heating at 55°C for 30 min. These data indicate that the β enzyme is more heat labile than the α enzyme. Both isozymes were stored in concentrated form in aliquots at -70°C as they were more labile in diluted form.

The estimation of molecular weight of two isozymes had been performed with a Sephacryl S-200 HR column from which AdoMet synthetase α and β were eluted as single

symmetrical peaks (Fig. 2). The apparent molecular weights had been estimated to be 91 kDa for isozyme α and 44 kDa for β isozyme. Markedly different molecular weights have been reported from mammalian sources such as sheep liver α 122 kDa; β_1 62kDa; β_2 70kDa (8); rat kidney γ form 190 kDa (9); rat liver β form 100 kDa (10); human lymphocyte enzyme 185 kDa (11); and human erythrocyte enzyme 74 kDa (12). Rechromatography of the individual forms did not lead to the re-appearance of the other form, which indicated that the two isoforms were not artifacts.

The effect of pH on the enzyme activity had been investigated in the pH range of 7.2-10.2. A pH optimum of 8.0 was found for AdoMet syntnetase α and the β enzyme exhibited a broad pH optimum of between 8.2 and 9.0 (Fig. 4).

The effect of divalent cations on the enzyme activity was studied. Maximal activity was obtained using Mg^{2+} for both α and β enzymes. Mn^{2+} could replace Mg^{2+} for both α and β enzymes with lower relative activity. The effect of other divalent cations such as Ca^{2+} and Fe^{2+} on α and β isozymes were distinct. Ca^{2+} gave no activity to the α enzyme while it gave 52% relative activity to the β enzyme. Fe^{2+} gave 30% relative activity to the α enzyme while it gave only 2% relative activity to the β enzyme (Fig. 5). This data also suggests that α and β enzymes are distinct isozymes.

Isoelectric focussing of Sephacryl S-200 HR purified isozymes on agarose slab gels revealed an isoelectric point of 4.7 for α isozyme and 5.6 for β isozyme, respectively. Only one gel band showed AdoMet synthetase activity for either α or β enzymes indicating that the two isozymes are not cross-contaminated.

The inhibitory effect of some amino acid analogues and dimethylsulfoxide on activities of both α and β AdoMet synthetase was studied. The results are summarized in Table 3 and Table 4. Among the compounds assayed, tripolyphosphate, which is a powerful inhibitor of AdoMet synthetase from other sources (9,13,14,15,16,17), was most active. L-cis-AMB, a methionine analogue, which was characterized as an inhibitor of both normal and tumorderived rat liver AdoMet synthetase (18,19,5) was also an effective inhibitor of both α and β isozymes. Cycloleucine, a known inhibitor of AdoMet synthetase from many sources (19,20), was less effective, with a higher IC_{50} value. On the other hand, dimethylsulfoxide, a known stimulator to mammalian AdoMet synthetase (6,8,9,21) gave a significant inhibition on α, and less on the β enzyme (Table 3). Other compounds tested, showing inhibition at higher concentrations or showing no inhibition, were listed in Table 4. Among the compounds tested, methapyrilene hydrochloride, an anti-histamine (22), showed some inhibition at 0.1 mM concentration. Other methionine analogues were found to be less effective inhibitors for both α and β AdoMet synthetase.

Multiple forms of AdoMet synthetase have been found in both eukaryotic and prokaryotic organisms (9,10,13,20,23,24). Our results suggest that we have isolated two distinct isoforms of AdoMet synthetase from *Leishmania mexicana* 227. Major differences between α and β isozymes are, respectively, 1) a molecular weight of 91 kDa vs. 44 kDa, 2) a distinct pH optimum of 8.0 vs. a broad pH optimum of between 8.2 and 9.0, 3) isoelectric points of 4.7 vs. 5.6, and 4) 58% α activity remained when heated at 55°C vs. no β activity remained. Whether or not the two isozymes of AdoMet synthetase in *Leishmania mexicana* represent two genetically distinct enzymatic proteins or represent the same gene product with post-translational modifications is an open question; as is the physiological significance of existence of two isoforms of the enzyme in this organism. Amino acid sequence analysis could clarify first question. We are attempting to purify two isozymes to homogeneity in order to perform amino acid sequence analysis in addition to enzyme kinetics studies, which may help us to determine if AdoMet synthetase activity of *Leishmania mexicana* can be regulated by substrates and/or product analogues.

Table 1. Partial purification of AdoMet synthetase α and β from *Leishmania mexicana* 227 promastigotes.

	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Purification (n-fold)
(1) Lysed Cells	2775	8625	3	100	1
(2) Crude Extract	504	6825	34	79	11
(3) Ammonium Sulfate Fractionation	495	33577	170	389	57
(4) DEAE-Cellulose	39	38250	2450	443	817
(5) Sephacryl S-200					
AdoMet Synthetase α	3	8934	7450	104	2483
AdoMet Synthetase β	1	2958	7250	34	2417

Table 2. Thermal Stability of AdoMet Synthetase α and β.

Storage Conditions	% Original Activity	
	<u>Isoform</u> α	<u>Isoform</u> β
Freezing (-70°C)		
6 days	100	100
4 months	85	69
Freezing (-20°C)		
6 days	100	100
4 months	77	57
Refrigeration (4°C)		
6 days	100	100
Heat (°C)		
45	87	34
55	58	0
65	4	--
75	0	--

The % original activities were determined by comparison with the enzymes, frozen at -70°C for up to 6 days. For heat treated enzymes, samples were heated for 30 minutes at indicated temperature before assay. The enzyme activities were determined in standard reaction mixture as described in Materials and Methods.

Table 3. Inhibition of AdoMet synthetase α and β by substrate and product analogues and dimethylsulfoxide.

Compound	IC_{50} (mM) Isoform α	IC_{50} (mM) Isoform β
Tripolyphosphate	0.12	0.10
L-*cis*-AMB	0.13	0.10
Cycloleucine	2.84	2.98
Dimethylsulfoxide	9% (v/v)	22% (v/v)

The enzyme activities were determined in the standard reaction mixture (see Materials and Methods) except each compound was included at various appropriate concentrations. The IC_{50} values were determined as the concentration of the compound that caused a 50% inhibition in activities relative to control assay.

Table 4. Compounds tested showing low inhibition or no inhibition of *Leishmania mexicana* AdoMet synthetase.

Compound	Concentration (mM)	Maximum Inhibition, α (%)	Maximum Inhibition, β (%)
Methapyrilene Hcl	0.1	28.0	10.0
S-Adenosyl-L-homocysteine	1.0	4.0	0.0
5-Azacytidine	2.5	0.0	0.0
DL-Homocysteine	10.0	42.0	33.0
DL-Homoserine	50.0	38.0	39.0
L-Homocysteine Thiolactone	50.0	16.0	51.0
O-Methyl-DL-serine	50.0	8.6	25.0

The enzyme activities were determined in the standard reaction mixture (see Materials and Methods) except each compound was included at various appropriate concentrations. Maximum percentage inhibitions were determined for the concentration of the compound that gave maximum inhibition of enzyme activities.

Figure 1. Chromatography of S-Adenosylmethionine Synthetase on DEAE-Cellulose

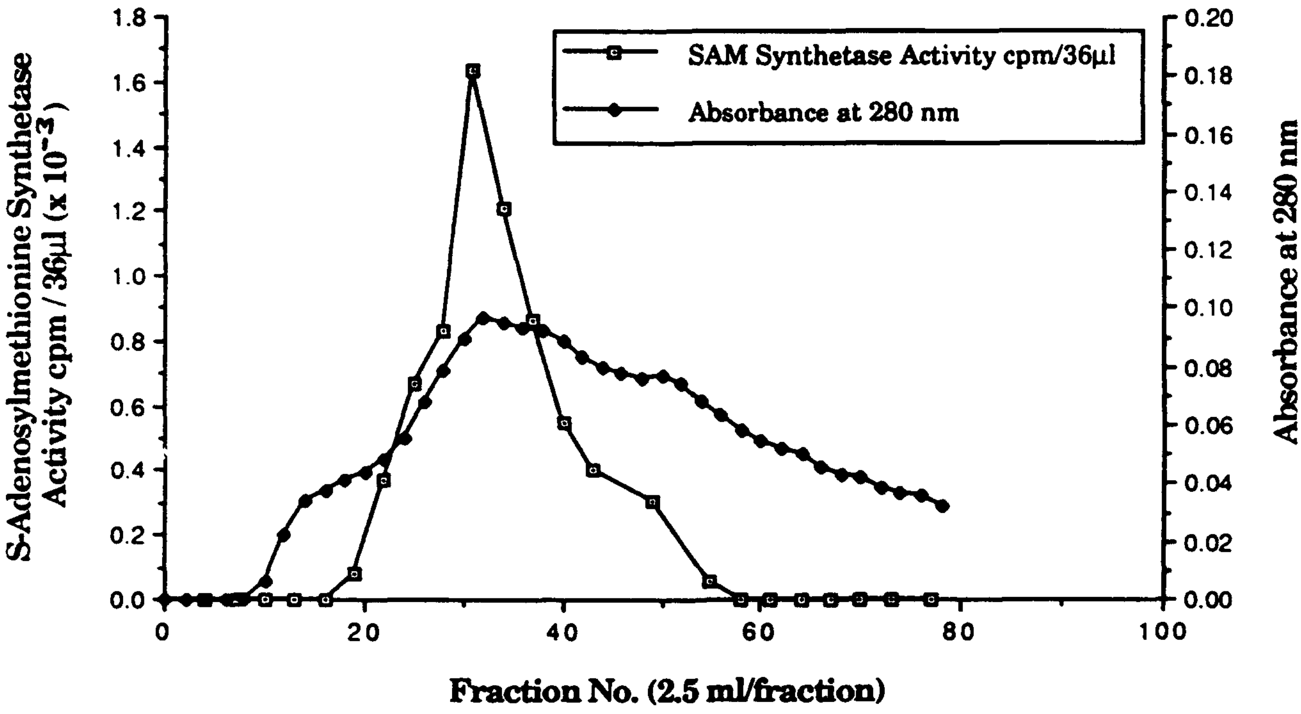


Figure 2. Elution Pattern of S-Adenosylmethionine Synthetase α and β from Sephacryl S-200 HR

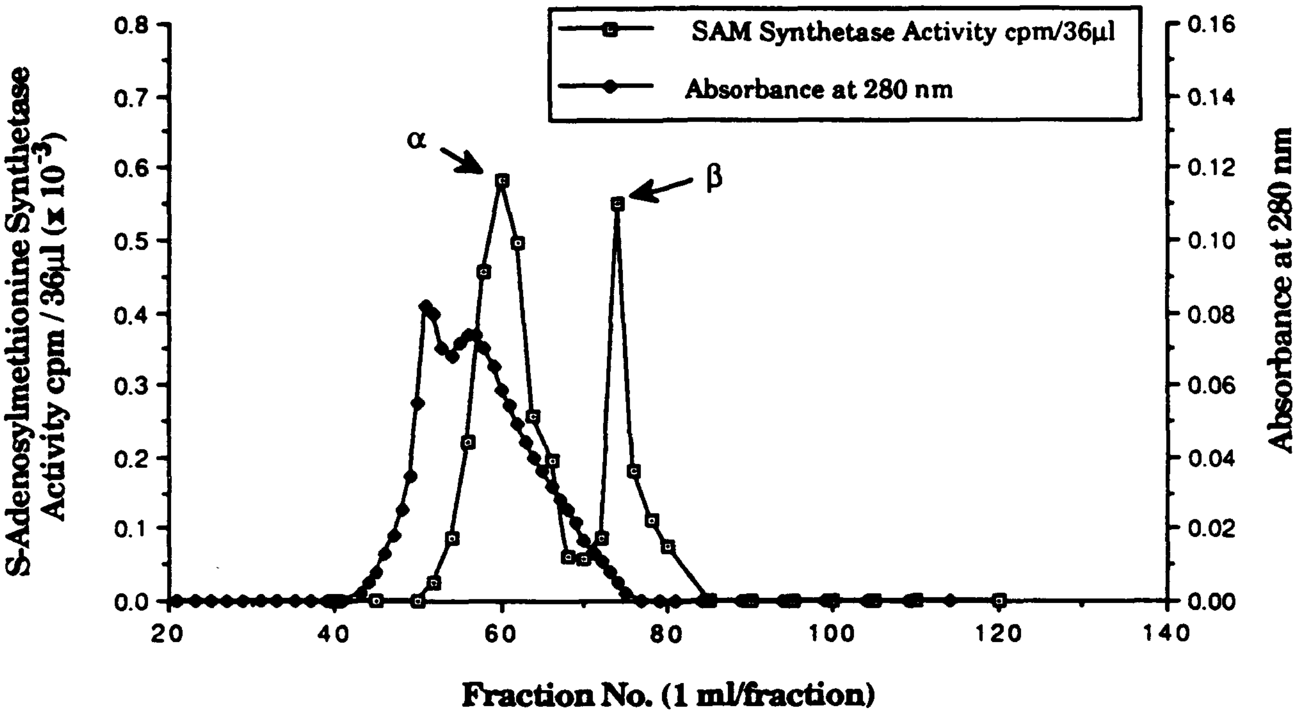


Figure 3. pH Optimum of α S-Adenosylmethionine Synthetase

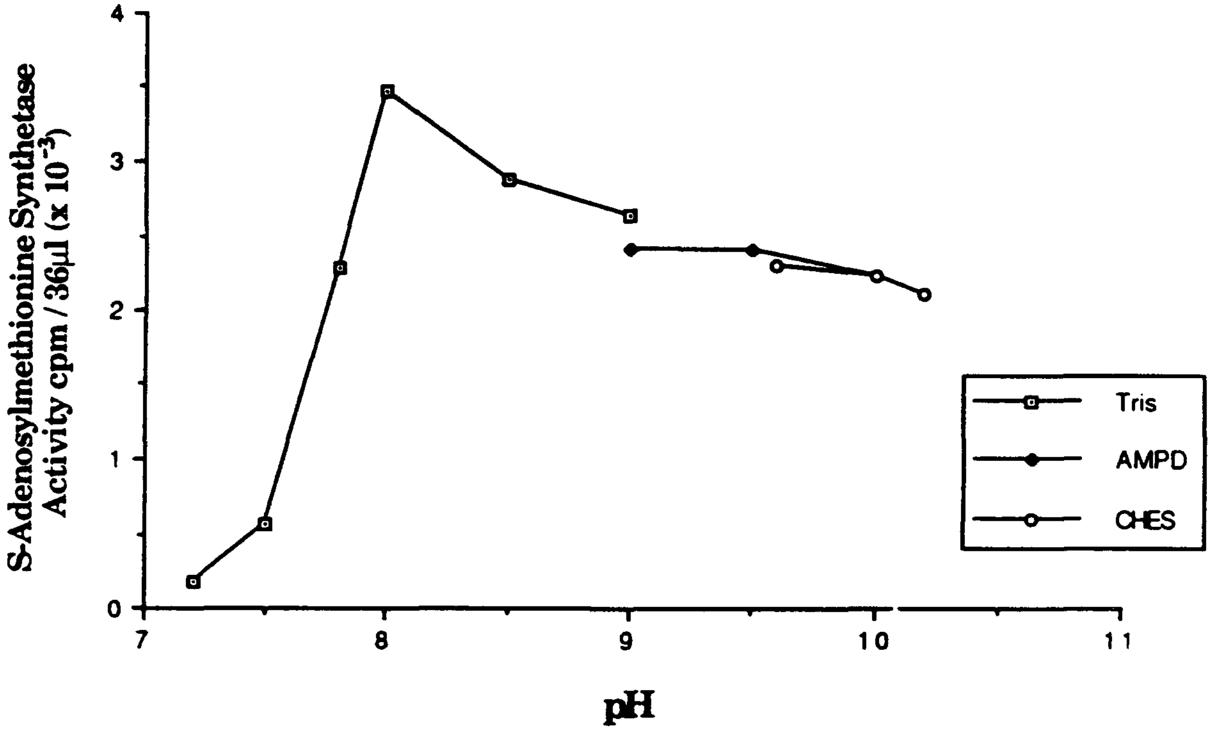


Figure 4. pH Optimum of β S-Adenosylmethionine Synthetase

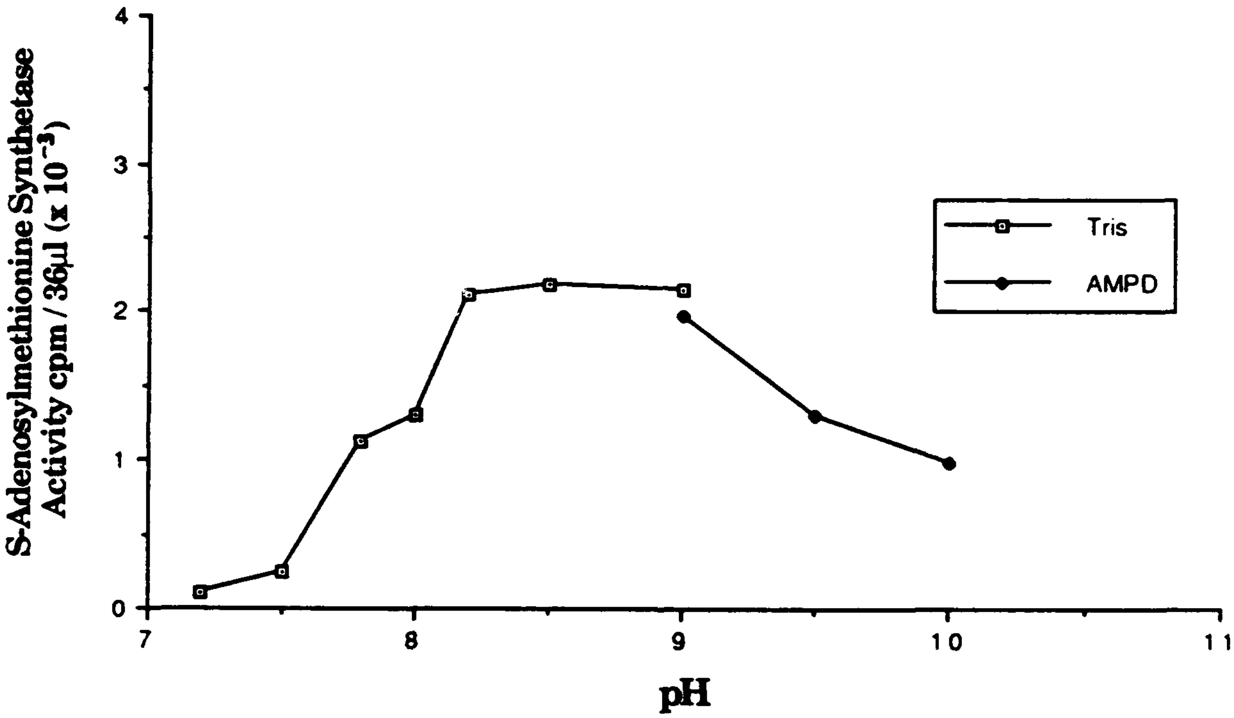
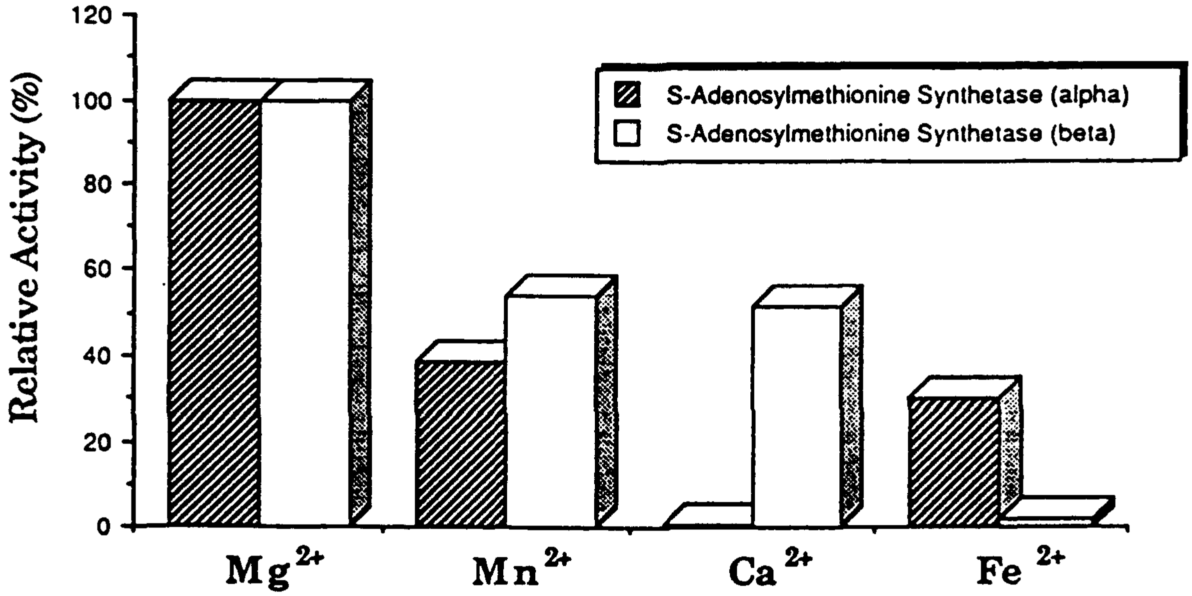


Fig. 5 Effect of Divalent Cations on α and β S-Adenosylmethionine Synthetase Activity



Assay in the presence of 20mM of each cation.

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